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14. ABSTRACT Chromosomal rearrangements and changes in copy number at various genomic loci are hallmarks of cancer cells and may be very early steps in tumorigenesis. The origins of genomic insults are poorly understood and this proposal aims to characterize one potential source of genomic instability, inappropriate DNA re-replication. In a normal eukaryotic cell cycle, the chromosomal DNA of a cell is replicated once, and only once, during S phase to ensure that each daughter cell receives exactly one complement of genomic material. By perturbing the regulation of several proteins involved in replication initiation, our laboratory has been able to conditionally induce varying amounts of re-replication in yeast cells. We have demonstrated that re-replication induces a rapid and significant decrease in cell viability and a cellular DNA damage response. We elected to focus our studies of genome instability on gene amplification because of its clinical importance in breast cancer. We have shown that re-replication is a potent inducer of gene amplification that generates structures similar to amplicons seen, but poorly understood, in tumors. The high frequency at which these amplification structures are generated is specific to re-replication, as similar structures are not observed when S phase DNA replication is impaired or DNA is directly damaged. We thus propose that re-replication arising from loss of replication control is a potential source of the genomic instability important for tumorigenesis.						
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Introduction

Genomic instability is common in breast cancer cells and can lead to loss of heterozygosity (Taback et al., 2003), gene amplifications (Lopez-Guerrero et al., 2003) and other genomic rearrangements. By inducing genomic alterations, genomic instability may promote carcinogenesis or make cancers more resistant to treatment. Currently, the source of genomic instability is unknown and this work aims to characterize one potential source of genomic instability, inappropriate DNA re-replication. In a normal eukaryotic cell cycle, the chromosomal DNA of a cell is replicated once, and only once, during S phase to ensure that each daughter cell receives exactly one complement of genomic material. By perturbing the regulation of several proteins involved in replication initiation, our laboratory has been able to conditionally induce varying amounts of re-replication in yeast cells. Effectively, cells enter, but do not complete, a second S phase (Nguyen et al., 2001), because only part of the genome re-replicates. The research supported by this grant is focused on understanding the consequences of such re-replication. Specifically, we have shown that re-replication leads to DNA damage and cell inviability. Most importantly, we have demonstrated that re-replication leads to genomic instability, in particular gene amplification. This re-replication induced gene amplification is specific to re-replication and does not occur with appreciable frequency after DNA damage or replication stress. Re-replication should thus be considered a potential source of the genomic instability seen in tumors.

Body

During the period from March 24th, 2004 to March 23rd, 2007, we completed a significant number of the tasks described in the initial application for this grant. In addition, we characterized a number of experimental systems that have been used to complete these tasks and published these characterizations.

During the course of this grant, I was a first author on two papers published in Molecular Biology of the Cell (Green and Li, 2005 and Green et al, 2006) which are attached as Appendix 1 and 2. We have submitted a third manuscript, on which I am the first author (Appendix 3), to Nature.

I presented work supported by this grant at four major conferences. I was also asked to give a talk at the Nucleic Acids Gordon Conference on June 6th, 2004 and present a poster at the Mechanisms of Genomic Integrity Conference on June 22nd, 2004 and the Era of Hope Meeting on June 9th, 2005. I was also asked to give a talk at the Eukaryotic DNA Replication Meeting at Cold Spring Harbor Laboratory, New York on September 9th, 2005.

Finally, I completed my dissertation during the time period of this grant. I will officially be granted my PhD at the end of the current quarter, but I have printed the dissertation and obtained all necessary signatures.

The overall purpose of the grant was to study the consequences of re-replication of cellular DNA. During a normal cell cycle, DNA replication is tightly controlled such that the genome is replicated once and only once before each mitosis. Loss of replication control has been proposed to be a source of the genomic instability that is associated with tumorigenesis. Our laboratory, and others, has elucidated many of the mechanisms that prevent re-replication from occurring. In doing so, we have established a yeast system with which we can induce re-replication in a population of cells arrested in metaphase. The work that has been supported by this grant is focused on understanding the consequences of this inappropriate DNA replication.

Task 1: Confirm that re-replication induces a cellular DNA damage response.

In the first reporting period, we completed task 1 of the initial grant application, which was to confirm that there was a DNA stress response as a consequence of re-replication. We additionally expanded our efforts in relation to this aim due to the surprising discovery that re-replication leads to a DNA damage response seemingly in the absence of the replication stress response. Much of this work was published in a manuscript described in prior annual reports (Green and Li, 2005).

Task 2: Establish whether pre-RC reformation, re-initiation or re-elongation induces the DNA damage response.

In task 2 of the initial grant application, we proposed to determine whether inappropriate pre-RC formation, re-initiation or re-elongation is the cause of the DNA damage observed when re-replication is induced. In prior annual reports, we demonstrated that inappropriate pre-RC formation is not sufficient to lead to DNA damage. We proposed to use hydroxyurea (HU), a ribonucleotide reductase inhibitor, to reduce re-elongation while still allowing re-initiation to occur in order to determine which of those two steps resulted in DNA damage. However, we have demonstrated that, surprisingly, re-initiation is markedly reduced when cells are treated with HU (data not shown) and thus we cannot prevent re-elongation without reducing re-initiation. We do not have an explanation for this result other than to note that the Brewer lab has reported at meetings that their genomic DNA replication assays suggest that, contrary to expectation, initiation in S phase is in fact reduced in the presence of HU (personal communication, B. Brewer). Although we have accomplished the first portion of task 2, we are thus unable to fully complete task 2 as described.

Task 3: Examine the structure of the DNA lesions induced by re-replication with electron microscopy.

My next task (task 3) was to use electron microscopy to determine the nature of DNA lesions induced by re-replication. Since re-replication initiation is required for DNA damage, as described above, it was likely that electron microscopy would be very useful to visualize the actual DNA lesions induced by re-replication. As described in prior annual reports, initial attempts to conduct these technically difficult experiments in our laboratory proved to be unfruitful. We consequently established collaboration, in principle, with Dr. Jose Sogo, the world's foremost expert on studying DNA lesions using electron microscopy. Unfortunately, Dr. Sogo was transitioning to a new university and was thus did not have access to an electron microscope during time periods when I could have visited.

Task 4: Search for double stranded break zones induced by re-replication.

We are very interested in determining where in the genome re-replication induced DNA damage occurs. If there are specific regions of increased damage we will attempt to correlate them with chromosomal features such as centromeres, cohesin binding sites and origins of re-replication. In the manuscript published during this reporting period (Green et al, 2006) we determine, on a genome wide level, the location of origins of DNA replication (Appendix 1, Figure 2). These data will be needed to correlate any regions of DNA damage with regions of re-replication.

Task 4 proposed the use of pulsed field gel electrophoresis to look for fragile zones where chromosomal breakage occurs as a consequence of re-replication. Despite several attempts and consultation with researchers who have used this technique, we have been unable to detect fragile zones (data not shown). The absence of a signal does not in any way demonstrate that these zones do not exist, as a limitation of this assay is that chromosomes currently re-replicating run aberrantly on the gel. Even if many molecules were broken in the same location, variable extents of re-replication would result in them

running at different locations on the gel. Consequently, we have decided to use another assay to look for regions of preferential damage.

Chromatin immunoprecipitation can be used to purify DNA that is bound by a protein of interest. It has been shown that Ddc2, a DNA damage response protein, localizes to sites of DNA damage (Melo et al, 2001) and I have demonstrated that Ddc2 sub-nuclear foci form when re-replication is induced (Green and Li, 2005). Dr. Katsu Shirahige is an expert at hybridizing DNA isolated using chromatin immunoprecipitation to DNA microarrays (Katou et al, 2003). This technology allows the precise determination of DNA binding sites of a protein across entire chromosomes. Hybridizing DNA immunoprecipitated using antibodies to Ddc2 will allow us to determine where DNA damage is occurring as damaged DNA will be bound to Ddc2 and thus enriched in the precipitated DNA.

We have established collaboration with Dr. Shirahige and have received strains and plasmids from his lab to conduct our experiments. We induce re-replication and perform the chromatin immunoprecipitation. We then send Dr. Shirahige the immunoprecipitated DNA and his lab amplifies, labels and hybridizes the DNA to microarrays. Initial experiments using conditions published by Dr. Shirahige's lab were quite successful (Figure 1), demonstrating our capability to conduct these experiments. However, attempts to use Ddc2 did not yield reliable results (data not shown). We suspect that this failure is due to the particular chromosome that Dr. Shirahige currently queries, chromosome VI. This chromosome re-replicates poorly (Green et al, 2006) so it is not surprising that re-replication dependent processes would be difficult to detect. Dr. Shirahige has now obtained reagents to query different chromosomes, including one that re-replicates very well. We anticipate that these new reagents would allow us to complete task 4.

Task 5: Establish whether re-replication leads to loss of heterozygosity.

Tasks 5 and 6 are focused on determining the long term consequences of re-replication on the stability of the genome. However, as we reported in prior annual reports, extensive re-replication leads to significant cell inviability (Green and Li, 2005). In order to study potential consequences of re-replication, we needed to establish a strain in which the cell death was reduced. We did this by perturbing two, rather than three, mechanisms that block re-replication. We have demonstrated that making these changes does result in reduced re-replication, in fact under some conditions, we are able to observe re-replication primarily from a single origin of replication.

The strain in which re-replication occurs primarily from a single origin of DNA replication will be used for tasks 5 and 6. Since demonstrating that re-replication occurs in this strain was essential before using it to study genomic instability, I delayed work on some of the tasks in my initial proposal in order to prepare this manuscript for publication. The manuscript has now been published in Molecular Biology of the Cell (Green et al, 2006) and is attached as Appendix 2 to this annual report.

In this study, we used microarray comparative genomic hybridization (CGH; Appendix 2, Figure 1) to provide a more comprehensive and detailed analysis of re-replication. Among other things, we demonstrate that re-replication can be induced within S phase (Appendix 2, Figure 3). We also show that it differs in amount and location from re-replication in G2/M phase, illustrating the dynamic nature of DNA replication controls (Appendix 2, Figures 2, 4 and 5). We note that re-replication occurs more readily during S phase, which could increase the likelihood that re-replication might occur in cells with fewer perturbations to cell cycle controls of DNA replication.

Finally, we show that very limited re-replication can be detected by microarray CGH when only two replication proteins are deregulated, suggesting that the mechanisms blocking re-replication are not redundant (Appendix 2, Figures 5, 6 and 7). The application of a more sensitive assay to re-replication allowed us to observe re-replication that had previously been undetectable. It seems more likely, therefore, that although cancer cells have not been observed to re-replicate, a more sensitive assay might be able to detect limited re-replication. Therefore we propose that eukaryotic re-replication at levels below current detection limits may be more prevalent and a greater source of genomic instability than previously appreciated.

We are thus prepared with the required strains to conduct task 5, but exciting results on task 6 have led us to focus on that task and switch the order of execution of tasks 5 and 6.

Task 6: Determine if re-replication promotes gene amplification.

In the initial grant application, we proposed to use a cassette of the ADH4 and CUP1 genes to select for gene amplification events. Although use of this system has been published before, there are a number of disadvantages of using this technique. The genetic modifications needed to use this system are complicated and time consuming to introduce and make the cells quite sick. Additionally, up to 4 to 7 copies of the cassette are required to grow under selective conditions, so this assay cannot detect the earliest and primary amplification event when a chromosomal region is first stably amplified from one to two copies. Consequently we decided to investigate whether we could develop a better system. We did, in fact, develop a new assay and complete this task. We have completed a manuscript describing this work and have submitted it to Nature (Appendix 3).

We have developed a new assay (Appendix 3, Figure 1C) that allows us to detect primary amplification events. In addition, the assay is versatile and allows the detection of other types of genomic rearrangements that result in heritable duplication of a chromosomal segment. We define a gene duplication event as one that results in two or more copies of a gene in a single cell, and a gene amplification as a gene duplication event that results in the two copies on a single DNA molecule. The assay is adapted from a colony color sectoring assay that can distinguish cells in a colony that have either 1 or

≥ 2 copies of ade3-2p, a hypomorphic allele of ADE3, based on whether the cells are pink or red, respectively (Koshland et al, 1985).

We can monitor the duplication of any locus in a cell lineage by inserting the ade3-2p reporter at that locus and looking for pink colonies with red sectors. Since all cells in a colony are derived from a single starting cell, a red sector indicates that one cell in the lineage has undergone a heritable increase in gene copy number. The greater the width of the sector, the earlier in the cell lineage this heritable change occurred. In preliminary experiments, we inserted a single copy of ade3-2p approximately 5 kb centromere-distal to ARS317 in the strain that re-replicates primarily from ARS317 (Appendix 2, Figure 7). After transiently inducing re-replication at a G2/M phase arrest with galactose induction of Δ ntcdc6-2A in liquid culture, we plated the cells and monitored the number of colonies with red sectors.

To identify heritable changes that occurred shortly after the re-replication insult, we monitored the frequency of sectors that comprise a half, quarter, or an eighth of the colony since these sectors reflect a single heritable event that occurs within the first three cell divisions in the colony. We observed that transient re-replication caused a 70-fold increase in the number of sectored colonies, indicating that re-replication triggers a robust increase in heritable gene duplication events that are easily detectable by this assay (data not shown).

For further genomic analysis of isolates identified by the sectoring assay, we have adapted high throughput microarray techniques developed by Christine Guthrie's lab here at UCSF. In our first trial we performed microarray CGH analyses on 49 isolates of which 43 had indeed duplicated the reporter, confirming the reliability of the sectoring assay. Of these, 39 had an extra copy of chromosome III, and 4 had an extra centromeric fragment of chromosome III. Whether the duplication of chromosome III is due to re-replication of the entire chromosome (which is only 350 kb in length) or, more interestingly, to re-replication induced chromosome nondisjunction remains an open question.

To reduce the likelihood of gene duplication due to re-replication of the entire chromosome, we created a cassette containing ARS317, ade3-2p and a selectable marker (Appendix 3, Figure 1A) and inserted it on several large chromosomes. Importantly, ARS317 still re-replicates when moved (Appendix 3, Figure 1B) but is not capable of re-replicating the entire chromosome.

We used this experimental system to demonstrate that re-replication leads to gene amplification and characterized those amplicons using microarray CGH, pulsed field gel electrophoresis and junction PCR analysis. Please see Appendix 3 for more complete description of this work, but the major results are summarized below. The amplified units, or amplicons, consist of large internal chromosomal segments up to several hundred kilobases long that are bounded by repetitive sequences and intrachromosomally arrayed in direct head-to-tail orientation. The high incidence of these segmental amplifications appears to be specific to re-replication, as they are not observed with

appreciable frequency when S phase DNA replication is impaired or DNA is directly damaged. While similarly arrayed amplicons in direct repeat have been observed in tumors (Albertson, 2006), these structures have eluded explanation by the prevailing breakage-fusion-bridge (BFB) model for gene amplification, which predicts formation of indirect repeats adjacent to telomeric deletions. We thus propose that loss of replication control should be considered a potential source of the genomic instability associated with carcinogenesis.

Key Research Accomplishments

Key accomplishments in the first reporting period:

We have demonstrated that re-replication leads to DNA damage and specifically, we have shown that:

- Extensive re-replication leads to significant cell inviability
- Re-replication leads to a *RAD9* and *RAD53* dependent metaphase arrest
- Ddc2-GFP foci form in the presence of re-replication
- Re-replication leads to Rad53p phosphorylation in a *RAD9* dependent manner
- Direct evidence of DNA double strand breaks can be observed after re-replication

The DNA damage response due to re-replication requires replication initiation

We have established strains in which re-replication is very limited – largely occurring from a single origin of DNA replication

Limited re-replication from these strains also induces a DNA damage response

Finally, we have demonstrated that cells are capable of surviving limited and transient re-replication, setting the stage for studying genomic instability in these cells

Key accomplishments in the second reporting period:

We have fully characterized strains in which re-replication is very limited

We have fully characterized the location and extend of re-replication in numerous re-replicating strains

We have demonstrated that re-replication can occur in S phase

We have adapted a single cell assay to screen for gene duplication events

We have demonstrated that re-replication leads to a significant increase in gene duplication events and have specifically:

- Confirmed the accuracy of this colony assay in detecting gene duplications
- Generated a protocol to perform high throughput microarray CGH
- Suggested that re-replication but not other cell cycle or chromosomal perturbations, can induce gene duplications
- Suggested that re-replication may specifically induce internal gene duplications (possibly representing a primary gene amplification event)
- Suggested ways to refine our screen to focus on internal gene duplications.

Key accomplishments in the third reporting period:

We have shown that origins that re-replicate can be transported to other regions in the genome and that they still remain the ability to reinitiate.

We have shown that re-replication leads to gene amplification, a stable duplication of a stretch of DNA

We characterize the amplified units, or amplicons, and showed they consist of large internal chromosomal segments up to several hundred kilobases long.

We have demonstrated that the amplicons are bounded by repetitive sequences and intrachromosomally arrayed in direct head-to-tail orientation.

The high incidence of these segmental amplifications appears to be specific to re-replication, as they are not observed with appreciable frequency when S phase DNA replication is impaired or DNA is directly damaged.

Re-replication might be an underappreciated mechanism that leads to the gene amplification that is closely associated with tumorigenesis.

Reportable Outcomes

The following are reportable outcomes from the first reporting period:

We have published a manuscript in Molecular Biology of the Cell describing some of the work supported by this grant (Green and Li, 2005, Appendix 1).

I presented this work in a talk and a poster at the Nucleic Acids Gordon Conference at Salve Regina University on June 6th, 2004.

I also presented this work at a poster presentation at the Mechanisms of Genomic Integrity Conference in Galway, Ireland on June 22nd, 2004.

This following are reportable outcomes for the second reporting period:

We have published a second manuscript in Molecular Biology of the Cell describing some of the work supported by this grant (Green et al, 2006, Appendix 2).

I presented this work in a talk at the Eukaryotic DNA Replication Meeting at Cold Spring Harbor Laboratory, New York entitled “Loss of re-replication control in *S. cerevisiae* results in extensive damage” on September 9th, 2005

The following are reportable outcomes from the third reporting period:

I have been awarded the degree of PhD.

Conclusions

I have made significant progress in regards to addressing the specific aims proposed in my initial application entitled, “DNA Damage and Genomic Instability Induced by Inappropriate DNA Re-replication.” We have published two papers on which I am a first author describing the results supported by this grant. One (Appendix 2, Green et al, 2006) was published during the second period and the other (Appendix 1, Green and Li, 2005) was published during the first reporting period. We have also prepared a manuscript that we have submitted to Nature (Appendix 3). I have also presented this work at four scientific conferences. At two of them I was asked to give a talk describing my work.

To maintain genome stability, the entire genome of a eukaryotic cell must be replicated once and only once per cell cycle. In many organisms, multiple overlapping mechanisms block re-replication, but the consequences of deregulating these mechanisms are poorly understood. I have shown that disrupting these controls in the budding yeast *Saccharomyces cerevisiae* rapidly blocks cell proliferation and leads to a significant DNA damage checkpoint response and DNA double strand breaks. These rapid and severe consequences suggest that even limited and sporadic re-replication could threaten the genome with significant damage.

We have also shown that limited re-replication can be induced when two mechanisms that block re-replication are deregulated. This has enabled us to establish a system in which the consequences of re-replication on genome stability can be studied. We have established an assay to study gene duplication events and have shown that re-replication does, in fact, lead to gene duplication events. The amplified units, or amplicons, consist of large internal chromosomal segments up to several hundred kilobases long that are bounded by repetitive sequences and intrachromosomally arrayed in direct head-to-tail orientation. The high incidence of these segmental amplifications appears to be specific to re-replication, as they are not observed with appreciable frequency when S phase DNA replication is impaired or DNA is directly damaged. Since most cancers, breast cancer included, show significant genomic instability, it is critical that we understand the source of such changes to the genome. We have made a great deal of progress during this grant period and have established numerous assays that will allow the lab to continue this important work.

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Loss of Rereplication Control in *Saccharomyces cerevisiae* Results in Extensive DNA Damage

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To maintain genome stability, the entire genome of a eukaryotic cell must be replicated once and only once per cell cycle. In many organisms, multiple overlapping mechanisms block rereplication, but the consequences of deregulating these mechanisms are poorly understood. Here, we show that disrupting these controls in the budding yeast *Saccharomyces cerevisiae* rapidly blocks cell proliferation. Rereplicating cells activate the classical DNA damage-induced checkpoint response, which depends on the BRCA1 C-terminus checkpoint protein Rad9. In contrast, Mrc1, a checkpoint protein required for recognition of replication stress, does not play a role in the response to rereplication. Strikingly, rereplicating cells accumulate subchromosomal DNA breakage products. These rapid and severe consequences suggest that even limited and sporadic rereplication could threaten the genome with significant damage. Hence, even subtle disruptions in the cell cycle regulation of DNA replication may predispose cells to the genomic instability associated with tumorigenesis.

INTRODUCTION

Eukaryotic DNA replication is tightly controlled such that every segment of the genome is replicated once and only once each cell cycle. This control is primarily exerted at the hundreds to thousands of replication origins where DNA replication initiates. Once an origin initiates in S phase, multiple mechanisms prevent it from reinitiating replication for the remainder of that cell cycle (Gopalakrishnan *et al.*, 2001; Nguyen *et al.*, 2001; Vas *et al.*, 2001; Yanow *et al.*, 2001; Vaziri *et al.*, 2003). Such tight control suggests that even an occasional reinitiation event would be deleterious to cells, and it is readily apparent that, in principle, excessive synthesis of just small segments of the genome could eventually threaten its stable propagation. Nonetheless, a direct analysis of the consequences of rereplication is needed to understand whether and how rereplication contributes to genomic instability. *S. cerevisiae* provides a powerful genetic system for such an analysis, especially as there is considerable understanding of both the mechanisms regulating replication and those protecting genome stability in this organism.

Eukaryotic replication initiation can be divided into two fundamental stages (reviewed in Bell and Dutta, 2002). In the first stage, which occurs in early G1 phase, a prereplicative complex (pre-RC) is assembled at replication origins through the sequential loading of the initiation proteins origin recognition complex (ORC), Cdc6, Cdt1, and Mcm2–7. In the second stage, activation of two kinases, Dbf4-Cdc7 kinase and a cyclin-dependent kinase (CDK), triggers events that culminate in replication initiation and disassembly of the prereplicative complex: additional replication proteins are recruited to the origin, the DNA is un-

wound, and replisomes are assembled at two nascent replication forks.

In addition to triggering initiation, CDKs also prevent reinitiation of eukaryotic DNA replication (Broek *et al.*, 1991; Dahmann *et al.*, 1995; Sauer *et al.*, 1995; Hua *et al.*, 1997). CDKs do this in part by down-regulating multiple components of the pre-RC, thereby preventing reassembly of these complexes at origins that have initiated. In budding yeast, CDKs promote the nuclear exclusion of Mcm2–7 (Labib *et al.*, 1999; Nguyen *et al.*, 2000), inhibit CDC6 transcription (Moll *et al.*, 1991) and promote its degradation (Drury *et al.*, 1997; Elsasser *et al.*, 1999; Drury *et al.*, 2000), and they seem to inactivate ORC through phosphorylation (Nguyen *et al.*, 2001). Making these three initiation factors refractory to CDK inhibition in metaphase-arrested cells allows a subset of origins to reinitiate and portions of the genome to rereplicate (Nguyen *et al.*, 2001). The limited extent of reinitiation suggests that not all inhibitory mechanisms to block rereplication have been identified. Consistent with this, a recent study indicates that CDK binding to ORC provides an additional mechanism to inhibit pre-RC formation (Wilmes *et al.*, 2004).

Analogous CDK-dependent mechanisms antagonizing Cdc6, ORC, and Cdt1 have been shown to inhibit rereplication in other eukaryotes (Jallepalli *et al.*, 1997; Lopez-Girona *et al.*, 1998; Nishitani *et al.*, 2000; Vas *et al.*, 2001; Wuarin *et al.*, 2002; Zhong *et al.*, 2003). Moreover, a CDK-independent mechanism to prevent pre-RC assembly has been identified in metazoans. Central to this mechanism is the protein Geminin (McGarry and Kirschner, 1998; Tada *et al.*, 2001; Wohlschlegel *et al.*, 2002), which binds to Cdt1 and is thought to sterically inhibit its ability to recruit Mcm proteins to replication origins (Lee *et al.*, 2004; Saxena *et al.*, 2004). Inactivation of geminin can lead to partial rereplication, confirming its role in preventing reinitiation of DNA replication (Quinn *et al.*, 2001; Mihaylov *et al.*, 2002; Melixetian *et al.*, 2004; Zhu *et al.*, 2004).

The partial extent of rereplication that we and others have observed suggests that these rereplicating forks are stalled

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or damaged before they can completely rereplicate the entire genome. Such insults to the rereplicating genome could trigger one or both of the checkpoint pathways that monitor genome integrity (reviewed in Melo and Toczyski, 2002; Nyberg *et al.*, 2002). The replication stress pathway responds to slowed or stalled replication forks, such as those arising from inhibition of nucleotide incorporation. The DNA damage pathway responds to chromosomal insults such as double-stranded breaks generated by ionizing radiation or enzymatic cleavage. These pathways activate proteins that stabilize stalled replication forks and repair DNA damage, respectively. In addition, they provide critical time to complete the replication or repair of DNA by imposing arrests at key cell cycle transitions.

Distinguishing whether the replication stress and/or DNA damage pathway is activated is an important first step in understanding the immediate molecular response to rereplication. This distinction is difficult because many checkpoint proteins and events are shared between the two pathways. For example, in metazoans, both types of genomic insults lead to the induction of p21, p53, and PIG3 protein levels; the phosphorylation of histone H2AX, p53, Cdc2, and the checkpoint kinases Chk1 and Chk2; and the organization of H2AX and Rad51 into subnuclear foci (Haaf *et al.*, 1995; Gottifredi *et al.*, 2001; Saintigny *et al.*, 2001; Ward and Chen, 2001; Brown and Baltimore, 2003). In a few of these responses, the kinetics or degree of change may vary between the two pathways, but overall the events considered to be hallmarks of DNA damage also are observed with replication stress. Complicating the distinction between these two responses is the potential for stalled forks to degenerate into damaged forks, particularly if the stalled forks are not properly stabilized (reviewed in Nyberg *et al.*, 2002).

Two groups have recently reported that the induction of rereplication in human cells induces a checkpoint response. The first group initially reported that rereplication induced by overexpression of Cdc6 and Cdt1 activates a DNA damage response (Vaziri *et al.*, 2003), but they have subsequently observed that overexpression of Cdc6 alone can induce this response in the absence of any detectable rereplication (Zhu *et al.*, 2004). Instead, they now report that rereplication induced by geminin depletion leads to what they suspect is a stalled fork response (Zhu *et al.*, 2004). Thus, they no longer assert that rereplication generates DNA damage. A second group observes similar events during geminin depletion, which they attribute to either a DNA damage or replication stress response (Melixetian *et al.*, 2004). Thus, although a clear assignment of pathways was not possible, the data are consistent with rereplication generating a replication stress-like response.

In *Saccharomyces cerevisiae*, the DNA damage and replication stress responses can be genetically distinguished, because the DNA damage pathway is primarily dependent on the BRCA1 C-terminus checkpoint protein Rad9p (reviewed in Toh and Lowndes, 2003), whereas the replication stress pathway is primarily dependent on Mrc1p (Alcasabas *et al.*, 2001; Osborn and Elledge, 2003). In this work, we take advantage of this genetic distinction to unambiguously determine which response is activated upon rereplication. We present evidence that rereplication leads to significant inviability and the activation of a RAD53 (budding yeast Chk2)-dependent checkpoint response. The *RAD9* dependence of the signaling pathway suggests that rereplication is triggering a DNA damage response and is not inducing a replication stress pathway. Moreover, we present the first direct evidence for the accumulation of chromosomal damage as a consequence of rereplication. These data indicate that rerep-

lication induces DNA damage and poses an immediate threat to both cell viability and genome integrity.

MATERIALS AND METHODS

Strain Construction

All strains (Table 1) with the exception of YJL310 were derived from YJL1737 (*orc2-cdk6A orc6-cdk4A leu2 ura3-52 trp1-289 ade2 ade3 bar1Δ::LEU2*). The *orc2-cdk6A* and *orc6-cdk4A* alleles encode mutant proteins in which alanine is substituted for the phosphoacceptor serines or threonines in CDK consensus phosphorylation sites (S/T-P-X-K/R). For *orc2-cdk6A*, residues 16, 24, 70, 174, 188, and 206 were mutated and for *orc6-cdk4A*, residues 106, 116, 123, and 146 were mutated. The following plasmids were digested and integrated as follows: pJL806 (*pGAL1 URA3/StuI*; Nguyen *et al.*, 2001), pJL1489 (*pGAL1-Δntcd6 URA3/StuI*; Nguyen *et al.*, 2001), pRS304-Rad53-HA-HIS (*RAD53-HA-HIS TRP1/HpaI*; Emili, 1998), YIp22 (*pMET3-HA3-CDC20 TRP1/MscI*; Uhmann *et al.*, 2000), and pBO1555 (*pMET3-HA3-CDC20 NatMX4/MscI*). pJL1206 (*MCM7-2NLS URA3/AspI*; Nguyen *et al.*, 2001) was used to replace *MCM7* with *MCM7-2NLS* by two-step gene replacement. The plasmid pBO1555 was generated by subcloning a *BglII* to *SalII* *pMET3-HA3-CDC20* fragment from YIp22 into pAG25 (Goldstein and McCusker, 1999) cut with *BglII* and *Sall*.

Genomic DNA from yJK7-2 (Melo *et al.*, 2001) was used as a template to generate a *DDC2-GFP kanMX* PCR fragment by using OJL1404 and OJL1405. Genomic DNA from U973 (*smf1Δ::TRP1 esr1-1*; Rothstein laboratory) was used as a template to generate a *smf1Δ::TRP1* polymerase chain reaction (PCR) fragment by using OJL1100 and OJL1111. Genomic DNA from the yeast haploid deletion collection (ResGen; Invitrogen, Carlsbad, CA) was used as a template to generate a *rad9Δ::kanMX* PCR fragment by using OJL1487 and OJL1488. The entire *RAD53* and *MRC1* open reading frames were deleted using PCR amplification of the *kanMX* from pAG25 with tagged primers by using the oligonucleotides indicated in Table 2 (Goldstein and McCusker, 1999).

Yeast Media

Cells were grown in YEP, synthetic complete (SC), or synthetic (S) broth medium (Guthrie and Fink, 1990) supplemented with 2% dextrose (wt/vol), 2% galactose (wt/vol), 3% raffinose (wt/vol), or 3% raffinose (wt/vol) + 0.05% dextrose (wt/vol). To obtain reproducible induction of rereplication, cells were inoculated from a culture containing 2% dextrose into a culture containing 3% raffinose + 0.05% dextrose and grown for 12–15 h overnight before the experiment commenced.

Cell Proliferation Assay

Yeast cells were diluted in S broth to OD₆₀₀ measurements of 0.2, and then serially diluted fivefold for six dilutions and spotted onto SDC-Ura or SGalC-Ura plates. For transient pulses of rereplication, cells grown overnight in SRaffC-Ura + 0.05% dextrose were pelleted and resuspended in YEPraff + 15 µg/ml nocodazole. Once >90% of the cells were arrested as large budded cells, galactose was added to a final concentration of 2%, and samples were removed at various time points, diluted in SD broth, and plated on SDC-Ura plates. Colonies were counted after 72 h at 30°C. All platings were done in triplicate, and two separate experiments were conducted. The mean and SE of the mean are shown. Statistical significance was determined using a Student's *t* test.

Flow Cytometry Analysis

Cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were pelleted and resuspended in YEPraff + 2 mM methionine to arrest cells in metaphase by Cdc20p depletion. Once arrested (>90% large budded cells), nocodazole (15 µg/ml) was added for an additional 30 min. Galactose was then added to a final concentration of 2%, and samples were taken every hour. Cells were fixed and stained with 1 µM Sytox Green (Molecular Probes, Eugene, OR) as described previously (Haase and Lew, 1997). Vertical lines indicate median DNA content after gating from 100 to 1000, which captures all whole, unclumped cells.

DDC2-Green Fluorescent Protein (GFP) Foci

Cells grown overnight in YEPraff + 0.05% dextrose were pelleted and resuspended in YEPraff + 15 µg/ml nocodazole. Once >90% of the cells were arrested as large budded cells, galactose was added to 2%, and samples were removed at various time points, washed twice in phosphate-buffered saline (PBS), and visualized using an Olympus BX60 microscope. Pictures were recorded using a Hamamatsu Orca-ER camera and OpenLab3.1.7 software. Fluorescent images were taken in three z sections that bracketed the thickness of the cell, and then projected into one image by using ImageJ's maximum pixel intensity function. Between 60 and 120 cells were scored for zero, one, or two or more foci per cell, for each strain for each time point. To obtain hydroxyurea (HU)-treated cells for the experiment in Figure 3A, cells were grown in YEPD. They were then arrested in G1 (>95% unbudded cells) with 50 ng/ml α factor and released into a HU arrest with the addition of pronase to a

Table 1. Strains used in this study

Strain	Source	Genotype
YJL310	Detweiler and Li (1998)	<i>leu2</i> -3112 <i>ura3</i> -52 <i>trp1</i> -289 <i>bar1Δ::LEU2</i>
YJL3244	Nguyen <i>et al.</i> (2001)	<i>orc2</i> - <i>cdk6A</i> <i>orc6</i> - <i>cdk4A</i> <i>leu2</i> <i>ura3</i> -52::(<i>pGAL1</i> , <i>URA3</i>) <i>trp1</i> -289 <i>ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::(pMET3-HA3-CDC20, TRP1)</i>
YJL3248	Nguyen <i>et al.</i> (2001)	<i>orc2</i> - <i>cdk6A</i> <i>orc6</i> - <i>cdk4A</i> <i>ura3</i> -52::(<i>pGAL1-Δntcdc6</i> , <i>URA3</i>) <i>trp1</i> -289 <i>leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::(pMET3-HA3-CDC20, TRP1)</i>
YJL3604	This study	<i>rad53Δ::kanMX6 sm1Δ::TRP1 orc2-cdk6A orc6-cdk4A ura3-52::(pGAL1-Δntcdc6, URA3) trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1)</i>
YJL3607	This study	<i>rad53Δ::kanMX6 sm1Δ::TRP1 orc2-cdk6A orc6-cdk4A ura3-52::(pGAL1-Δntcdc6, URA3) trp1-289 ura3-52::(pGAL1, URA3) trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1)</i>
YJL5048	This study	<i>orc2-cdk6A orc6-cdk4A ura3-52::(pGAL1-Δntcdc6, URA3) trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1)</i>
YJL5055	This study	<i>orc2-cdk6A orc6-cdk4A leu2 ura3-52::(pGAL1, URA3) trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1)</i>
YJL5060	This study	<i>orc2-cdk6A orc6-cdk4A ura3-52::(pGAL1-Δntcdc6, URA3) trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1) rad9Δ::kanMX</i>
YJL5065	This study	<i>orc2-cdk6A orc6-cdk4A leu2 ura3-52::(pGAL1, URA3) trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1) rad9Δ::kanMX</i>
YJL5085	This study	<i>orc2-cdk6A orc6-cdk4A ura3-52::(pGAL1, URA3) trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1) mrc1Δ::kanMX</i>
YJL5087	This study	<i>orc2-cdk6A orc6-cdk4A ura3-52::(pGAL1-Δntcdc6, URA3) trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1) mrc1Δ::kanMX</i>
YJL5132	This study	<i>orc2-cdk6A orc6-cdk4A leu2 ura3-52::(pGAL1, URA3) trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 ddc2::(DDC2-GFP, kanMX)</i>
YJL5135	This study	<i>orc2-cdk6A orc6-cdk4A leu2 ura3-52::(pGAL1-Δntcdc6, URA3) trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 ddc2::(DDC2-GFP, kanMX)</i>
YJL5408	This study	<i>rad53Δ::kanMX6 sm1Δ::TRP1 orc2-cdk6A orc6-cdk4A ura3-52::(pGAL1-Δntcdc6, URA3) trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1) rad9Δ::kanMX cdc20::(pMET3-HA3-CDC20, NatMX)</i>
YJL5411	This study	<i>orc2-cdk6A orc6-cdk4A ura3-52::(pGAL1-Δntcdc6, URA3) trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1) rad9Δ::kanMX cdc20::(pMET3-HA3-CDC20, NatMX)</i>
YJL5441	This study	<i>orc2-cdk6A orc6-cdk4A ura3-52::(pGAL1-Δntcdc6, URA3) trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 rad53::(RAD53-2HA6HIS, TRP1) mrc1Δ::kanMX cdc20::(pMET3-HA3-CDC20, NatMX)</i>

final concentration of 100 µg/ml and HU to a final concentration of 0.2 M. Samples were processed for quantification as described above. To obtain phleomycin-treated cells for the experiment in Figure 3A, cells were grown in YEPD, arrested with 15 µg/ml nocodazole (>95% large budded cells), and then treated with phleomycin at a final concentration of 20 µg/ml (Cayla, Toulouse, France). Samples were processed for quantification as described above.

Rad53p Immunoblot

Cells grown overnight in SRaffC-Ura + 0.05% dextrose were pelleted and resuspended in YEPraff + 15 µg/ml nocodazole. Once >90% of the cells were arrested as large budded cells, galactose was added to a final concentration of 2%, and samples were removed at various time points. Cells (8.5 ml) at OD₆₀₀ 0.5-1.0 were pelleted and lysed by vortex mixing and boiling with 300 µl of 0.5-mm glass beads (Biospec Products, Bartlesville, OK) and 300 µl of SDS-PAGE loading buffer [8% glycerol (vol/vol), 100 mM Tris-HCl, pH 6.8, 1.6% SDS (wt/vol), 1.6 × 10⁻³% bromphenol blue (wt/vol), 100 mM dithiothreitol, and 1 mM phenyl-

methylsulfonyl fluoride] with protease inhibitors (1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml chymostatin, and 1 mM benzamidine) and phosphatase inhibitors (1 mM Na₃VO₄, 50 mM NaF, and 50 mM Na β-glycerophosphate). The soluble protein was quantified using a Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard (Sigma-Aldrich, St. Louis, MO). Forty micrograms of each protein sample was electrophoresed on a 7.5% SDS-PAGE gel and transferred to nitrocellulose (Protran BA85; Applied Scientific, San Francisco, CA). The membrane was probed with anti-HA 16B12 (Covance, Berkeley, CA) at 1:1000, followed by sheep anti-mouse horseradish peroxidase (NA931V; Amersham Biosciences, Piscataway, NJ) at 1:2000. Immunoblots were developed with the SuperSignal system (Pierce Chemical, Rockford, IL).

Assaying Induction of a Metaphase Arrest

Cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were pelleted and resuspended in YEPraff + 2 mM methionine to arrest cells in metaphase by Cdc20p depletion. Once arrested (>90% large budded cells), galactose was

Table 2. Oligonucleotides used in this study

Oligo	Purpose	Sequence
OJL1404	<i>DDC2-GFP</i>	AAAGGTACGTGGGACAAGAC
OJL1405	<i>DDC2-GFP</i>	AGACAGCAACACATCTAG
OJL1110	<i>sm1Δ</i>	ctcgcatcgatAAGGATCACGTTCTTCTGC
OJL1111	<i>sm1Δ</i>	gtagaccccgAGAACATTGCGGGTTCAAG
OJL1002	<i>rad53Δ</i>	GAGAGAAATAGTGAGAAAAGATACTGTTACACAAACATCAACggatccccgggttaattaa
OJL1003	<i>rad53Δ</i>	ctttaaaaaggggcagcatttctatgggtatgtcctgaattcgagctgttaaac
OJL1487	<i>rad9Δ</i>	GCTCCCCATCAAATAAGGTC
OJL1488	<i>rad9Δ</i>	TATGTGCGCCAGTACTC
OJL1497	<i>mrc1Δ</i>	AGACAAACAACTAAGGAAGTCGTTATTGCGTTTGAACCTATCACCAAATTTAGTG- cgatccccgggttaattaa
OJL1498	<i>mrc1Δ</i>	CGACTACTTCAAGACAGCTCTGGAGTTCAACTCTTCGGAAAAGATAAAAAACCA- catcgatgttcggatcgctcg

added to a final concentration of 2% for 2 h, and then the cells were filtered and washed with S broth and resuspended in SGaC-Met,Ura + 50 ng/ml α factor. Samples were fixed in 67% ethanol (vol/vol), washed twice with PBS, and resuspended in 50 ng/ml 4'-6-diamidino-2-phenylindole (DAPI). Cells were visualized by fluorescence microscopy on an Olympus BX60 microscope and quantified as pre- or postmetaphase based on nuclear morphology. At least 200 cells were scored for each strain for each time point, and the experiment was executed twice. The mean percentage of postmetaphase cells and the SE of the mean from the two experiments are charted.

Pulsed Field Gel Electrophoresis (PFGE)

YJL3244 and YJL3248 cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were pelleted and resuspended in YEPRaft + 2 mM methionine to arrest cells in metaphase by Cdc20p depletion. Once arrested (>90% large budded cells), nocodazole was added to a final concentration of 15 μ g/ml for 30 min, after which galactose was added to a final concentration of 2% at time 0. To obtain HU-treated cells for the experiment in Figure 5, cells were grown in YEPD. They were then arrested in G1 (>95% unbudded cells) with 50 ng/ml α factor and released into a HU arrest with the addition of pronase to a final concentration of 100 μ g/ml and HU to a final concentration of 0.2 M. To obtain phleomycin-treated cells for the experiment in Figure 5, cells were grown in YEPD, arrested with 15 μ g/ml nocodazole (>95% large budded cells), and then treated with phleomycin at a final concentration of 20 or 200 μ g/ml (Cayla).

To make plugs for PFGE, 6×10^8 cells were washed twice with ice-cold 50 mM EDTA and resuspended to 500 μ l with 50°C SCE (1 M sorbitol, 0.1 M Na citrate, and 10 mM EDTA). Lyticase was added to a final concentration of 150 U/ml, and 250 μ l of the sample was mixed with 250 μ l of molten, 50°C 1% SeaPlaque GTG LMP agarose (FMC Bioproducts, Rockland, ME), and then aliquoted into disposal plug molds (170-3713; Bio-Rad). The plug molds were allowed to solidify at 4°C, and then placed in SCEM + lyticase [1 M sorbitol, 0.1 M Na citrate, 10 mM EDTA, 5% β -mercaptoethanol (vol/vol), and 160 U/ml lyticase] for 24 h at 37°C. Plugs were then washed three times in T₁₀E₁ (10 mM Tris, pH 8.0, and 1 mM EDTA) for 15 min each wash and resuspended in proteinase K solution [1% sarcosyl (wt/vol), 0.5 M EDTA, and 2 mg/ml proteinase K] for 48 h at 55°C. Finally, plugs were washed three times in T₁₀E₁ for 15 min each wash and left overnight at 37°C in T₁₀E₁, which removes background fluorescence during ethidium bromide visualization of the gel.

Plugs were cut in half and loaded on a 1% SeaKem LE agarose (wt/vol) gel in 0.5× TBE (45 mM Tris, 45 mM borate, and 1 mM EDTA). The gel was electrophoresed in 14°C 0.5× TBE on a CHEF DR-III system with initial switch time of 50 s, final switch time of 90 s, run time of 22 h, voltage of 6 V, and angle of 120°. The gel was stained with 0.5 μ g/ml ethidium bromide in 0.5× TBE for 1.5 h, destained in deionized water for 2 h, and imaged with an AlphaImager. The DNA was then nicked in 0.5 M HCl for 1 h, denatured in 1.5 M NaCl, 0.5 M NaOH for 40 min, and neutralized in 3M NaCl, 55 mM Tris base, 455 mM Tris-HCl for 40 min. The DNA was then transferred to a GeneScreen Plus nylon membrane and cross-linked with 0.12 J of UV light in a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The membrane was probed with an ARS305 fragment (Nguyen *et al.*, 2001) and imaged and quantified with a Storm 840 (Amersham Biosciences).

RESULTS

Rereplication Rapidly Blocks Cell Proliferation

Previous work in our laboratory established yeast strains in which rereplication can be induced in metaphase-arrested cells (Nguyen *et al.*, 2001). These yeast strains contain genetic alterations that make three replication initiation proteins refractory to the inhibitory effect of the CDK Cdc28p. The CDK phosphorylation of two subunits of the origin recognition complex, Orc2p and Orc6p, was blocked by mutating their CDK consensus phosphorylation sites (*orc2-6A*, *orc6-4A*). Cdc28p-directed nuclear exclusion of the Mcm2-7p complex (Labib *et al.*, 1999; Nguyen *et al.*, 2000) was prevented by fusing two tandem copies of the simian virus 40 nuclear localization signal to Mcm7p (MCM7-2NLS). Finally, CDK regulation of Cdc6p was disrupted by integrating *pGAL1-Δntcdc6*, which expresses an N-terminally truncated and slightly stabilized Cdc6p (Δ ntcdc6p), under the control of the galactose-inducible *GAL1* promoter (Drury *et al.*, 1997). In this rereplicating strain, rereplication is detectable only after Δ ntcdc6p is induced by growth in galactose-containing medium. A parallel strain, containing *pGAL1* instead of *pGAL1-Δntcdc6*, does not rereplicate and serves as a negative control strain (Figure 1A).

Further characterization of these strains initially revealed that sustained rereplication leads to a dramatic decrease in plating efficiency (Figure 1B). Both the *pGAL1-Δntcdc6* rereplicating strain and *pGAL1* control strain grew with similar efficiency when plated on medium containing dextrose, which represses the *pGAL1* promoter. However, when cells were plated on medium containing galactose, the *pGAL1-Δntcdc6* rereplicating strain showed a decrease in plating efficiency by at least three orders of magnitude. In the absence of perturbations of ORC and MCM, expression of Δ ntcdc6p had no effect on cell growth as assayed by colony size or plating efficiency on galactose-containing medium (our unpublished data).

Significant inhibition of cell proliferation also could be seen with transient induction of rereplication (Figure 1C). Both the *pGAL1-Δntcdc6* rereplicating strain and *pGAL1* control strain were arrested in metaphase with nocodazole then exposed to galactose to induce rereplication. After varying amounts of time in galactose, cells were plated on dextrose-containing medium to assess the number of cells that could give rise to viable colonies (colony-forming units). Because Δ ntcdc6p becomes undetectable within 30 min after galactose-induced cells are repressed by the addition of dextrose (Nguyen *et al.*, 2001), we expected reinitiation to end after cell plating. The *pGAL1* control strain showed only a slight decrease in colony-forming units after 3 h in galactose. In contrast, the *pGAL1-Δntcdc6* rereplicating strain showed a fivefold decrease in colony-forming units after only 30 min in galactose and a nearly 50-fold decrease after 3 h, a statistically significant difference ($p < 0.002$).

Rereplication Induces a RAD53-dependent Metaphase Checkpoint Arrest

To determine how rapidly rereplicating cells cease dividing, we examined cells microscopically 2 d after transient exposure to galactose. Most rereplicating cells that did not give rise to colonies also did not rebud (our unpublished data), indicating that the cells could not progress beyond the G1 commitment point of the next cell cycle. To pinpoint where in the cell cycle these cells were blocked, we arrested cells in metaphase by depleting them of Cdc20p, which is required for the metaphase-anaphase transition (Schwab *et al.*, 1997; Visintin *et al.*, 1997), induced rereplication with galactose for 2 h, and then restored Cdc20p expression to remove the original metaphase block. α Factor was added to trap any cells that progressed into G1 phase of the next cell cycle (Figure 2A). Cell and nuclear morphology were used to distinguish between cells that were in metaphase and cells that were postmetaphase (anaphase/telophase or G1 phase). More than 90% of the *pGAL1*-negative control cells proceeded past metaphase and accumulated in G1 phase. In contrast, <20% of the *pGAL1-Δntcdc6*-rereplicating cells had exited metaphase 5 h after Cdc20p expression was restored. Similar results were obtained when these cells were monitored after rereplication was induced for only 1 h instead of 2 h (our unpublished data). Because rereplication was barely detectable by flow cytometry after 1 h of induction (Figure 1A), these data suggest that even limited rereplication induces a metaphase arrest.

In budding yeast, genotoxic stresses such as replication fork stalls or DNA damage induce a metaphase arrest that requires activation of the checkpoint kinase Rad53p (Allen *et al.*, 1994; Weinert *et al.*, 1994; Sanchez *et al.*, 1996; Sun *et al.*, 1996), the homolog of Chk2 in mammalian cells and Cds1 in *Schizosaccharomyces pombe*. To determine whether rereplication might activate these pathways, we induced rereplication in a *rad53Δ* mutant background and monitored the

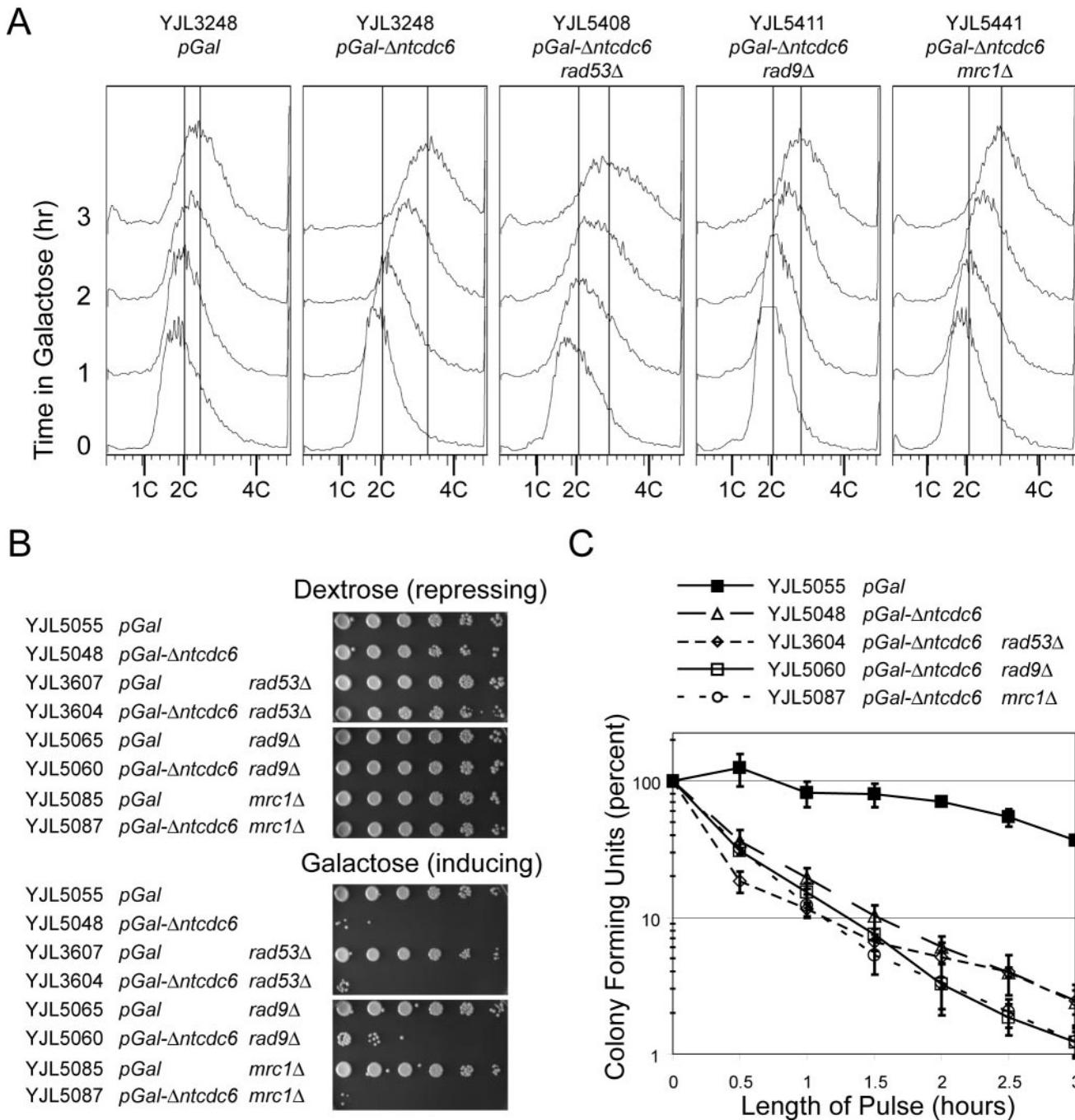
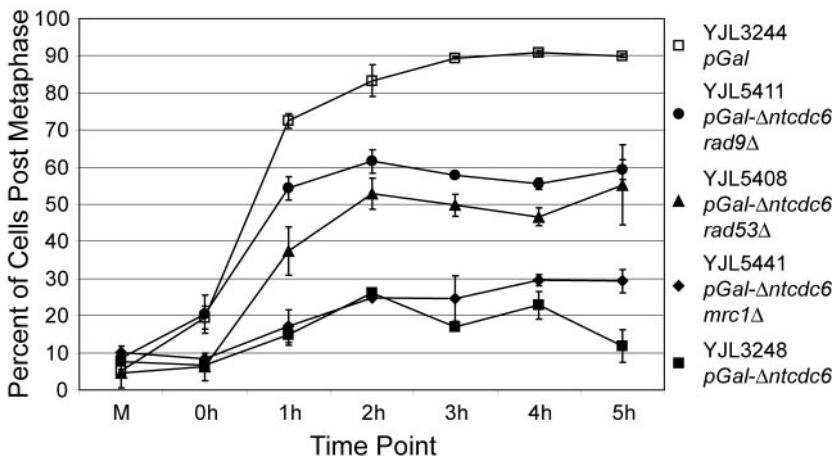
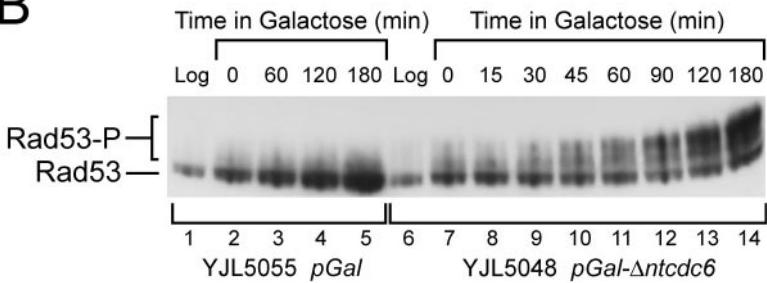


Figure 1. Induction of rereplication rapidly blocks cell proliferation. (A) Checkpoint-deficient strains are capable of rereplicating. Cells with the indicated genotypes plus *pMET3-HA3-CDC20 orc2-cdk6A orc6-cdk4A MCM7-2NLS* were grown in medium containing 3% raffinose + 0.05% dextrose. Metaphase arrest was induced by adding 2 mM methionine, to transcriptionally deplete Cdc20p, and 15 µg/ml nocodazole. Then, 2% galactose was added, and samples were taken hourly for flow cytometry. Vertical lines indicate the median DNA content for the 0- and 3-h time points. (B) Constitutive induction of rereplication prevents cell proliferation. Cells with the indicated genotypes plus *orc2-cdk6A orc6-cdk4A MCM7-2NLS* were grown on plates containing 2% dextrose and serially diluted into S broth with fivefold dilutions. The dilutions were plated on medium containing either 2% dextrose, which represses rereplication, or 2% galactose, which induces rereplication in strains containing *pGAL1-Δntcdc6*. (C) Transient induction of rereplication rapidly inhibits colony forming potential. Cells with the indicated genotypes plus *orc2-cdk6A orc6-cdk4A MCM7-2NLS* were grown in medium containing 3% raffinose plus 0.05% dextrose and arrested in metaphase with addition of 15 µg/ml nocodazole. Galactose (2%) was added for the indicated number of hours to allow for transient induction of rereplication and cells were then plated on medium containing 2% dextrose to score colony-forming units (CFU). For each strain, the CFU is expressed as a percentage of the CFU present at time 0 h. Error bars show SE of the mean from two experiments.

A**B**

ability of these cells to progress past metaphase. Flow cytometry demonstrated that rereplication was still induced in the presence of the *rad53Δ* mutation (Figure 1A), and vital staining with phloxine B showed that most of the cells remained metabolically alive after 3 h of induction (our unpublished data). The percentage of cells that could complete metaphase, however, increased from <20% to nearly 50%. This result suggests that a significant portion of the checkpoint-proficient rereplicating cells were arrested solely in response to a *RAD53*-dependent checkpoint. The remaining 50% of the cells also seemed to activate this checkpoint (see below) but presumably stayed arrested because they were subjected to an additional *RAD53*-independent metaphase block (see *Discussion*).

Additional evidence that rereplication activates a *RAD53*-dependent checkpoint response was obtained by examining Rad53p directly. Activation of Rad53p protein kinase is tightly correlated with its hyperphosphorylation (Allen *et al.*, 1994; Weinert *et al.*, 1994; Sanchez *et al.*, 1996; Sun *et al.*, 1996), a modification that retards Rad53p mobility during gel electrophoresis. After inducing rereplication with galactose in metaphase-arrested cells, we monitored the phosphorylation state of Rad53p by immunoblotting total cell lysates (Figure 2B). In the *pGAL1* control strain, Rad53p remained hypophosphorylated for the duration of the galactose induction, consistent with the absence of any checkpoint arrest of the cell cycle. In the *pGAL1-Δntcdc6*-rereplicating strain, however, Rad53p hyperphosphorylation was detected within 45 min of induction, and the majority of the protein became hyperphosphorylated by 120 min. Together, the metaphase arrest and Rad53p hyperphosphorylation indicate that Rad53p is activated as part of a checkpoint response triggered by rereplication. The nearly complete con-

Figure 2. Rereplication induces a *RAD53*-dependent checkpoint response. (A) Rereplication induces a metaphase arrest that is dependent in part on *RAD53* and *RAD9*. Cells with the indicated genotypes plus *pMET3-HA3-CDC20 orc2-cdk6A orc6-cdk4A MCM7-2NLS* were arrested in metaphase by transcriptional depletion of Cdc20p in medium containing 3% raffinose and 2 mM methionine (M). Then, 2% galactose was added for 2 h to allow the induction of rereplication followed, at time 0 h, by release from the Cdc20p depletion arrest by transfer of cells to medium lacking methionine but containing 2% galactose and α factor. At hourly intervals after the release, DAPI-stained cells were scored ($n = 300$) as pre- or postmetaphase. The percentage of postmetaphase cells is shown for each strain, along with the SE of the mean. (B) Rereplication induces phosphorylation of Rad53p. Cells containing the indicated genotypes plus *RAD53-HA orc2-cdk6A orc6-cdk4A MCM7-2NLS* were grown in medium containing 3% raffinose + 0.05% dextrose and arrested in metaphase with 15 μg/ml nocodazole. Then, 2% galactose was added to allow the induction of rereplication, and at the indicated times samples were harvested for immunoblot analysis of Rad53p-HA. The hypophosphorylated protein is indicated by Rad53 and the hyperphosphorylated protein is indicated by Rad53-P.

version of Rad53p to the hyperphosphorylated form (Figures 2B and 4A) further suggests that this response was activated in almost all rereplicating cells.

Rereplication Induces Formation of *Ddc2-GFP* Foci

Because the genome is only partial rereplicated in our strains, many rereplication forks cannot be properly terminating with a converging fork from the adjacent replicon. This suggests that many of the rereplication forks must be stalled or disrupted, potentially signaling replication stress, DNA damage, or both. Analysis of the Ddc2p response to rereplication provided an initial hint that rereplication elicits a checkpoint response to DNA damage. Like Rad53p, Ddc2p is required for the response to both DNA damage and replication stress. Ddc2p in complex with Mec1p is recruited to both sites of double-strand breaks (Kondo *et al.*, 2001; Melo *et al.*, 2001) and stalled replication forks (Katou *et al.*, 2003; Osborn and Elledge, 2003) as part of the sensing of these lesions by the checkpoint pathways. Previous studies established that Ddc2p relocates from a diffuse nuclear distribution to punctate subnuclear foci in response to DNA damage (Melo *et al.*, 2001). We observed that similar foci are not generated in response to HU in our strains, thereby providing a possible way to distinguish between the two responses (Figure 3A).

This distinction was demonstrated in a *pGAL1-Δntcdc6*-rereplicating strain where *DDC2* was replaced by *DDC2-GFP*. Initial experiments were performed in dextrose-containing medium to ensure tight repression of *pGAL1-Δntcdc6*. The rereplicating strain was arrested in metaphase with nocodazole, exposed to 20 μg/ml of the DNA damaging agent phleomycin, and examined by fluorescence mi-

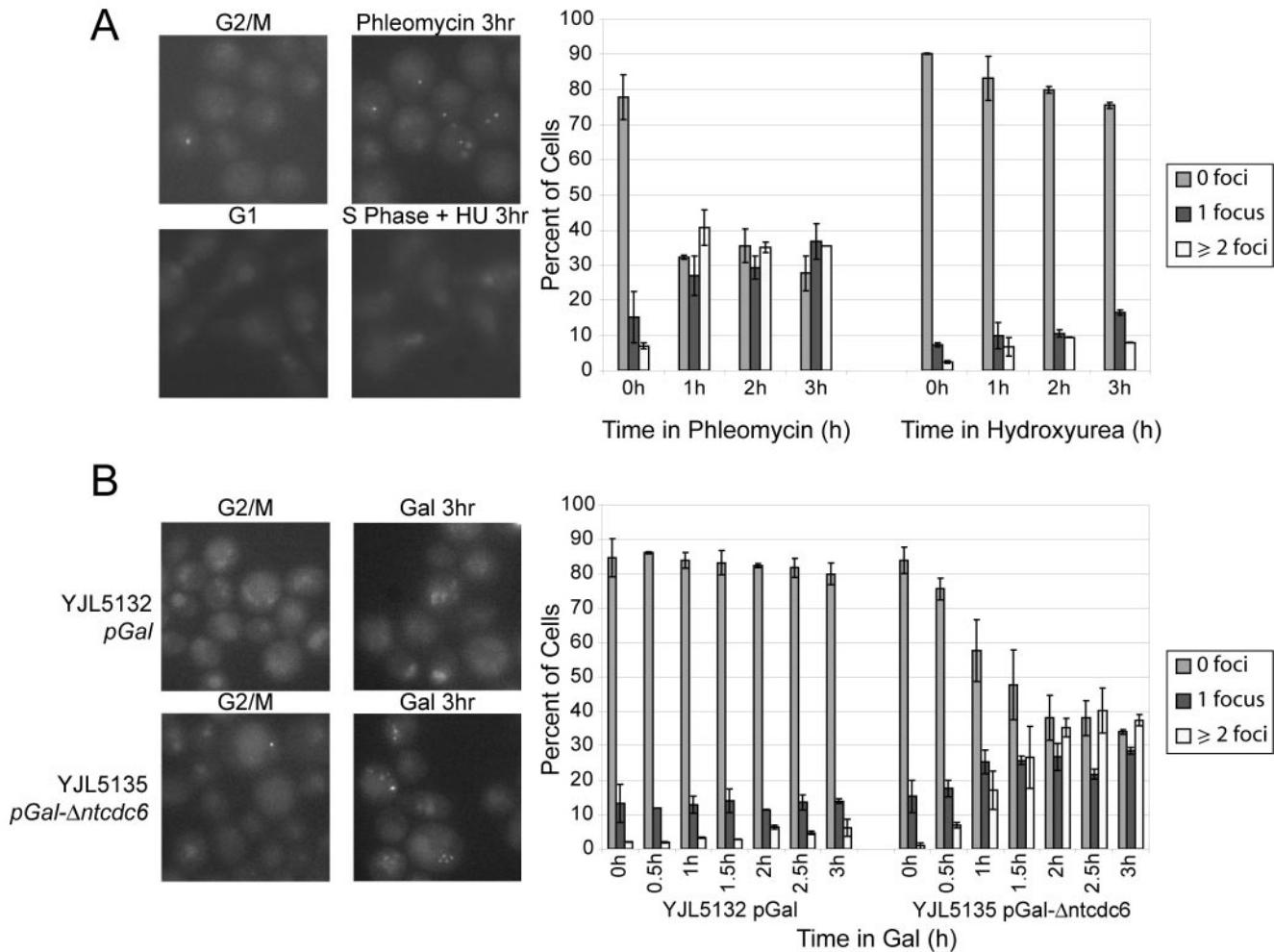


Figure 3. Subnuclear Ddc2p foci consistent with DNA damage are formed when rereplication is induced. (A) HU-induced replication stress does not induce subnuclear Ddc2p foci to the same extent as DNA damage. YJL5135 (*ddc2:DDC2-GFP pGAL1-Δntcdc6 orc2-cdk6A orc6-cdk4A MCM7-2NLS*) growing in medium containing 2% dextrose was arrested in metaphase with 15 µg/ml nocodazole followed by treatment with 20 µg/ml phleomycin to induce DNA damage. A parallel culture was arrested in G1 phase with α factor and released from the arrest into 0.2 M HU to induce replication stress. At hourly intervals after either phleomycin addition or release into HU, cells were scored for subnuclear GFP foci, and the number of cells with zero foci, one focus, or two or more foci was quantified. Representative images at 0 and 3 h are shown. Error bars show SE of the mean from two experiments ($n = 60\text{--}120$ per experiment). (B) Rereplication induces Ddc2p foci. YJL5135 and YJL5132 (*ddc2:DDC2-GFP pGAL1 orc2-cdk6A orc6-cdk4A MCM7-2NLS*) growing in medium containing 3% raffinose + 0.05% dextrose were arrested in metaphase by the addition of 15 µg/ml nocodazole. Then, 2% galactose was added to induce rereplication in YJL5135 and at 30-min intervals the number of foci per cell was quantified ($n = 60\text{--}120$ per experiment). Representative images and quantification are shown as in A.

croscopy. Within 1 h, one or more subnuclear foci of Ddc2p-GFP were observed in most cells (Figure 3A), consistent with previously published observations. In contrast, when these cells were released from a G1 arrest into S phase in the presence of 0.2 M HU, there was little induction of Ddc2p-GFP subnuclear foci even 3 h after imposition of the replication block (Figure 3A). If phleomycin is added to these cells, subnuclear Ddc2p-GFP foci occur within an hour, indicating that damage-induced foci are observable in HU-arrested cells (our unpublished data). Similar results were observed in wild-type cells not containing any perturbations of ORC, Mcm2–7, or Cdc6.

To examine the localization of Ddc2p after rereplication, the *pGAL1-Δntcdc6* rereplicating and *pGAL1* control strains containing *DDC2-GFP* were arrested in metaphase, induced with galactose, and examined at 30-min intervals by fluorescence microscopy. In the *pGAL1-Δntcdc6* strain, within 1 h

of induction of rereplication, there was a significant increase in Ddc2p-GFP subnuclear foci (Figure 3B). Within 2 h, the number of cells with foci and the number of foci per cell were quantitatively similar to the response observed with the addition of the DNA damaging agent phleomycin. Little increase in Ddc2p-GFP foci was observed in the *pGAL1* control strain. Thus, these findings suggest that rereplication induces a DNA damage checkpoint.

Rereplication Induces a DNA Damage Response

For a more definitive examination of whether rereplication was triggering a DNA damage response, a replication stress response, or both, we took advantage of the genetic distinction between these two checkpoint pathways in budding yeast. Both pathways converge on *RAD53* and induce a metaphase arrest. However, upstream of *RAD53*, the DNA

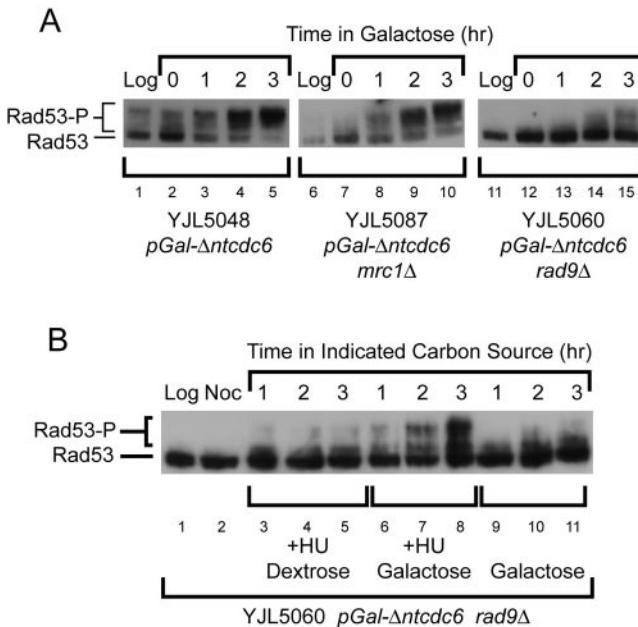


Figure 4. The checkpoint response induced by rereplication is dependent on Rad9p and not Mrc1p. (A) Cells with the indicated genotypes plus *orc2-cdk6A* *orc6-cdk4A* *MCM7-2NLS* were grown in 3% raffinose + 0.05% dextrose and arrested in metaphase by the addition of 15 µg/ml nocodazole. Then, 2% galactose was added, and at the indicated times samples were harvested for immunoblot analysis of Rad53p-HA. The hypophosphorylated protein is indicated by Rad53 and the hyperphosphorylated protein is indicated by Rad53-P. (B) The *rad9Δ* cells are capable of responding to stalled rereplication forks. YJL5060 (*rad9Δ pGAL1-Δntcdc6 orc2-cdk6A orc6-cdk4A MCM7-2NLS*) grown in medium containing 3% raffinose + 0.05% dextrose was arrested at metaphase with 15 µg/ml nocodazole and split into three cultures: 0.2 M HU and 2% dextrose were added to the first culture; 0.2 M HU and 2% galactose were added to the second; and 2% galactose was added to the third. Immunoblot analysis was performed as described in A.

damage response is predominantly dependent on *RAD9*, whereas the replication stress response is predominantly dependent on *MRC1*. We individually deleted each gene in the *pGAL1-Δntcdc6* rereplicating strain and the *pGAL1* control strain and investigated whether the metaphase arrest and Rad53p hyperphosphorylation induced by rereplication was dependent on either gene. Initial experiments established that rereplication was still induced on all chromosomes in the *mrc1Δ* and *rad9Δ* mutants (Figure 1A; our unpublished data).

As described above, the proportion of cells arrested in metaphase due to rereplication was approximately halved when *RAD9* was deleted. A slightly higher reduction was observed when *RAD9* was deleted, whereas a much smaller reduction was observed upon deletion of *MRC1* (Figure 2A). Thus, nearly half of the rereplicating cells that are arrested in metaphase are solely held at that arrest by a *RAD9*-dependent pathway. The remainder, as discussed previously, seem to be subjected to an additional metaphase block. The hyperphosphorylation of Rad53p induced during rereplication (Figure 4A, lanes 1–5) also was dramatically reduced in a *rad9Δ* mutant background (Figure 4A, lanes 11–15). The simplest interpretation of these results is that the Rad53 phosphorylation and *RAD9*-dependent metaphase arrest induced by rereplication is primarily triggered through the *RAD9*-dependent DNA damage response pathway.

The virtually complete dependence of Rad53p hyperphosphorylation on *RAD9* suggests that rereplication generates little or no *MRC1*-dependent signaling of replication stress. Alternatively, one could hypothesize that the *rad9Δ* mutation, the metaphase state of the cell, or an insufficient number of rereplicating forks, somehow prevents the detection of replication stress in our rereplicating cells. For example, if Mrc1p did not properly assemble onto rereplication forks during reinitiation as it normally does at replication forks during normal initiation, the *rad9Δ* cells would be unable to signal the presence of stalled forks.

To demonstrate that we can indeed detect replication stress during rereplication in a *rad9Δ* mutant, the mutant strain was arrested in metaphase, split into three separate culture conditions, and each harvested for immunoblot analysis of Rad53p. Galactose was added to one culture to induce rereplication. As described above, there was little Rad53p hyperphosphorylation because of the *rad9Δ* mutation (Figure 4B, lanes 9–11). Galactose and HU were added to a second culture to induce replication stress during rereplication. In these cells, robust Rad53p hyperphosphorylation could now be observed (Figure 4B, lanes 6–8), presumably through activation of the *MRC1*-dependent replication stress response pathway. Finally, dextrose and HU were added to the third culture. Dextrose represses the *pGAL1* promoter and stifles any induction of rereplication. No Rad53p hyperphosphorylation was observed in this culture (Figure 4B, lanes 3–5), confirming that rereplication forks were generating the HU-induced replication stress response observed in the second culture. Thus, the *MRC1*-dependent replication stress response pathway is capable of sensing stalled rereplication forks during a metaphase arrest in a *rad9Δ* background. The lack of any significant activation of this pathway in the absence of HU suggests that stalled rereplication forks are not triggering the checkpoint response observed in rereplicating cells. Consistent with this conclusion is the observation that the extent and kinetics of Rad53p hyperphosphorylation induced by rereplication are unchanged by deletion of *MRC1* (Figure 4A, lanes 6–10). Together, our data suggest that DNA damage, and not replication stress, is the predominant genotoxic insult accumulating as a consequence of rereplication.

Rereplication Induces Double-stranded Breaks

Given the induction of a DNA damage response, we looked for direct evidence of DNA damage induced by rereplication. We assayed whether rereplication results in double-stranded breaks by monitoring the appearance of subchromosomal fragments by PFGE. To verify that PFGE can detect chromosome fragmentation, we examined yeast chromosomes from metaphase-arrested cells treated with phleomycin, which generates double-stranded breaks. At high doses of phleomycin, all chromosomes were converted to a heterogeneous pool of subchromosomal fragments (Figure 5A, lanes 4–6). These results were confirmed by Southern blot analysis of these gels, by using *ARS305* to probe for chromosome III (Figure 5A, lanes 15–17).

Similar chromosome fragmentation was not observed in cells arrested in S phase with HU (Figure 5A, lanes 1–3 and 12–14). Replicating structures, such as replication bubbles and forks, are thought to significantly retard DNA mobility during PFGE, and whole chromosomes with many replicating structures are retained in gel loading wells (Hennessy *et al.*, 1990). Nonetheless, the absence of any significant subchromosomal fragments even after prolonged HU arrest suggests that there is no rapid or widespread degeneration of stressed replication forks to double-stranded breaks.

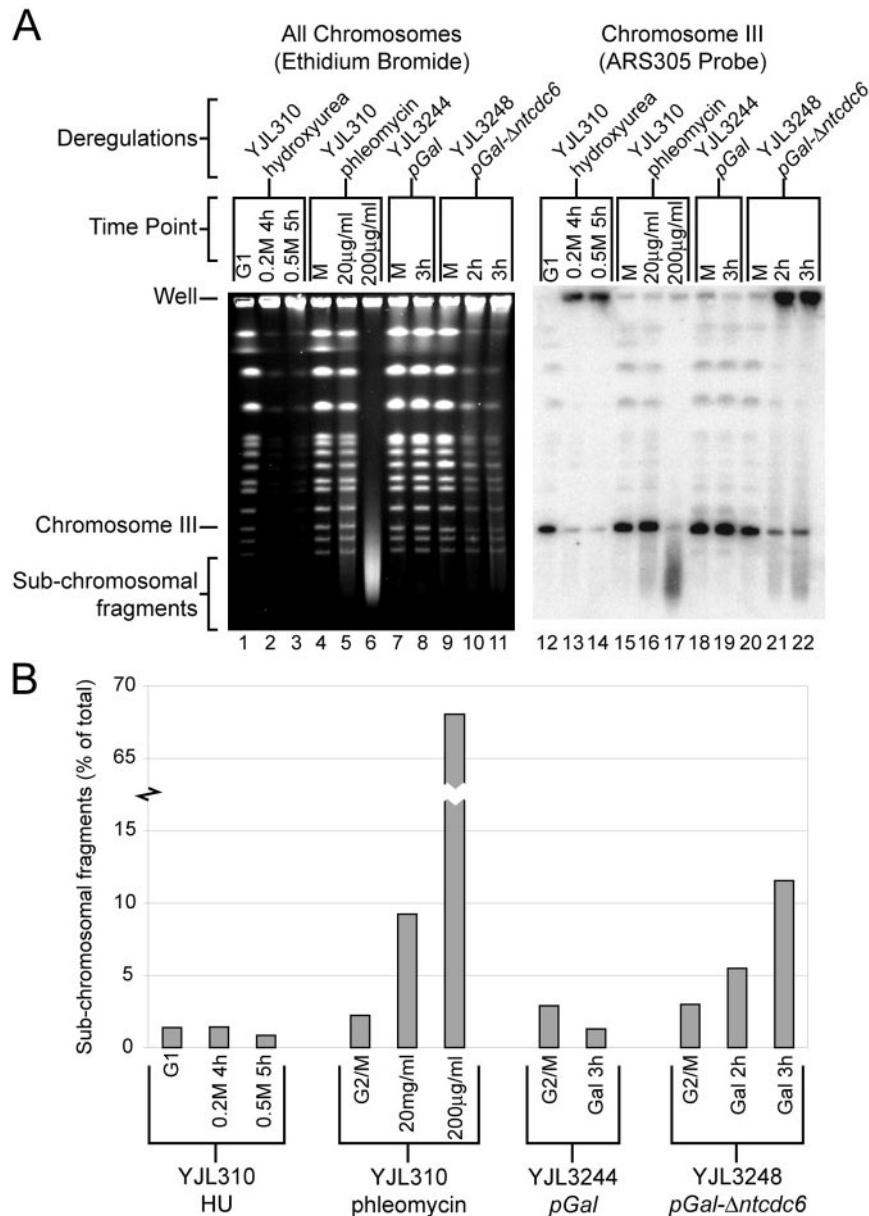


Figure 5. Rereplication induces double-stranded breaks. (A) Rereplication generates subchromosomal fragments. (Lanes 1–11) PFGE stained with ethidium bromide. (Lanes 12–22) Southern blot of PFGE probed with *ARS305* fragment to detect chromosome III. (Lanes 1–3, 12–14) YJL310 (*CDC6 ORC2 ORC6 MCM7*) was arrested in G1 phase with α factor and then released from the arrest into the indicated amounts of HU for the indicated times. (Lanes 4–6, 15–17) YJL310 was arrested in metaphase with 15 μ g/ml nocodazole and then treated for 2 h with the indicated amount of phleomycin. (Lanes 7–8, 18–19) YJL3244 (*pGAL1 orc2- cdk6A orc6- cdk4A MCM7-2NLS pMET3-HA3-CDC20*) was arrested in metaphase in medium containing 3% raffinose and 2 mM methionine. Once arrested, galactose was added to 2%. (Lanes 9–11, 20–22) YJL3248 (*pGAL1-Δntcdc6 orc2- cdk6A orc6- cdk4A MCM7-2NLS pMET3-HA3-CDC20*) was treated as for YJL3244. DNA from equivalent numbers of cells were loaded in each lane, except twice as many cells were loaded in lanes 1–3 and 12–14 to compensate for their G1 or nearly G1 DNA content. (B) Quantification of subchromosomal fragment from Southern blot in A. The intensity of subchromosomal signal is shown as a percentage of the total signal for each lane.

Like HU treatment, rereplication caused the majority of each chromosome to be retained in the wells. However, rereplication also generated subchromosomal fragments, which looked like a smear of DNA migrating from below the smallest chromosome up toward the well (Figure 5A, lanes 9–11). This could be seen more clearly by Southern blot analysis, which showed an accumulation of chromosome III fragments migrating faster than the smallest full-length chromosome (Figure 5A, lanes 20–22) in amounts comparable with those generated by 20 μ g/ml phleomycin (Figure 5B). This induction of subchromosomal fragments was specific to rereplicating cells, because no such induction was seen in the control strain (Figure 5A, lanes 18–19). Similar subchromosomal fragments were observed when the Southern blots were probed for chromosome 4 and 7 (our unpublished data). Thus, rereplication, but not replication stressed by HU, generates double-stranded DNA breaks.

Checkpoint Responses Do Not Reduce the Lethality Induced by Rereplication

By mobilizing a corrective response and delaying the cell cycle, checkpoint pathways help to protect cells from insults that would disrupt the proper transmission of genetic information. In some cases, however, recovery from the insult may not be possible despite the activation of a checkpoint. For example, degradation of Mcm proteins in the middle of S phase disrupts active replication forks and seems to activate the replication stress response: Rad53p is hyperphosphorylated and cells experience a *RAD9*-independent metaphase arrest (Labib *et al.*, 2001). Despite the activation of this checkpoint, cells are unable to recover their ability to replicate after Mcm proteins are restored (Labib *et al.*, 2001), presumably because Mcm proteins cannot be reloaded onto the disrupted replication forks. To determine whether the DNA damage response is able to protect cells from the amount and type of DNA lesions generated by rereplication,

we examined the viability of rereplicating cells that harbor deletions in *RAD53*, *RAD9*, or *MRC1*. Strains deleted for any of these genes showed similar decreases in viability as checkpoint-proficient strains when subjected to constitutive or transient ($p > 0.35$ at 3 h) rereplication (Figure 1, B and C). This suggests that the extent of rereplication in these cells generates an amount or type of lethal genotoxic stress that is irreparable.

DISCUSSION

Eukaryotic cells use multiple overlapping mechanisms to prohibit reinitiation of DNA replication within a single cell cycle. An obvious reason why cells might impose such extensive and layered safeguards is that even a low frequency and amount of extra DNA synthesis could eventually alter genome content. We report here that rereplication can induce an immediate and severe threat to the cell. Rereplicating cells rapidly and permanently cease cell division. They phosphorylate Rad53p in a *RAD9*-dependent manner and arrest in metaphase. This checkpoint response is unlikely to be a novel "rereplication checkpoint." Rather, we infer from the stereotypical DNA damage response that rereplication rapidly generates DNA lesions that are recognized by the cell as DNA damage. Thus, the use of multiple mechanisms to prevent rereplication not only preserves genome content in the long-term but also protects cells from lethal genomic insults in the short-term.

Surprisingly, we have been able to demonstrate that replication triggers little or no replication stress response, even though rereplication forks fail to complete a full round of replication. The Rad53p phosphorylation observed during rereplication was almost exclusively dependent on *RAD9*, which signals DNA damage, and was independent of *MRC1*, which signals replication stress. Similarly, the metaphase arrest induced by rereplication was more dependent on *RAD9* than on *MRC1*. Importantly, the absence of a replication stress response was not due to an inability to respond to replication stress. In a *rad9Δ* mutant background, where rereplication by itself failed to induce Rad53p phosphorylation, the addition of HU to stress the rereplicating forks leads to robust and persistent Rad53p phosphorylation. The simplest interpretation of these data is that rereplicating forks fail to complete a full round of replication, not because they eventually stall, but because they somehow degenerate into DNA lesions that are recognized as DNA damage. These results contrast with those obtained in human cells depleted of geminin, where the resulting rereplication can be associated with the replication stress response (Melixetian *et al.*, 2004; Zhu *et al.*, 2004). Whether these contrasting results reflect differences in species or protocol for inducing rereplication remains to be addressed in the future.

A key question raised by these findings is how rereplication generates DNA lesions without inducing a stalled fork response. Because a prompt DNA damage response is observed in almost all cells in the presence of the microtubule depolymerizing agent nocodazole, the lesions are unlikely to be a consequence of spindle tension on partially replicated chromosomes. Consistent with this, we can induce rereplication and observe the attendant DNA damage response during S phase (our unpublished data), suggesting that a mitotic state is not required to generate the lesion. Moreover, preliminary evidence suggests that elongation is restrained during rereplication (our unpublished data), raising the possibility that rereplicating replisomes encounter problems

that could lead to DNA lesions. We therefore suspect that the lesions are generated by the act of rereplication itself.

Any molecular model for how these lesions are generated must explain why they are generated during rereplication and not during normal replication. One possible explanation is that the first round of replication structurally alters chromosomes in a manner that interferes with their rereplication within the same cell cycle; sister chromatid cohesion, which is established during DNA replication, provides precedence for such a replication-coupled change in chromosome state (reviewed in Nasmyth, 2001). Other possible explanations include hypothetical problems specific to rereplication such as poor coordination of histone synthesis and/or nucleosome assembly with rereplication (Verreault, 2003), rereplicating forks from later rounds of rereplication overtaking rereplicating forks from earlier rounds, or defective assembly of replisomes during reinitiation.

An important approach to understanding how rereplication generates DNA damage is to characterize the molecular structure of the primary lesions that are induced. Importantly, these primary lesions may not be the chromosomal breaks that we observed by PFGE. Other abnormal DNA structures that could trigger the DNA damage response might be generated earlier before degenerating into chromosomal breaks. Fork collapse, for example, can generate "chicken feet" structures (Sogo *et al.*, 2002), which expose free double-stranded DNA ends without cleaving the chromosome. Further analysis of rereplicating DNA will hopefully yield more insight into the structure of these primary lesions and the molecular mechanisms by which they are generated.

Although rereplication induces a *RAD9*-dependent checkpoint response, this response offers little protection against the lethal consequences of rereplication (Figure 1B). This lack of protection is reminiscent of the futile induction of a *RAD9*-independent checkpoint response after complete Mcm degradation in S phase (Labib *et al.*, 2001). Loss of Mcm proteins from replication forks is apparently irreparable even after resynthesis of the proteins, because there is no efficient mechanism to reload Mcm proteins at forks. Similarly, in our rereplicating cells the damage induced by replication may be irreparable and overwhelm any possible protective effect of the DNA damage response. Additionally, other lethal problems may arise from rereplication that are not dependent on DNA damage and cannot be corrected by the DNA damage response. Such additional problems might account for the partial persistence of metaphase-arrested cells when rereplication is induced in the absence of *RAD53* or *RAD9* (Figure 2A). Fully understanding the lethal consequences of rereplication will require further molecular characterization of the terminal phenotype of rereplicating cells.

The extra copies of genes that are generated by rereplication have long been considered a possible source of genomic instability. Our observation that DNA damage is generated during rereplication suggests an additional way by which rereplication might generate genomic changes. Interestingly, in mammalian cells, overexpression of a single replication initiation protein Cdt1 can induce subtle rereplication (Vaziri *et al.*, 2003) and has been implicated in tumorigenesis (Arentson *et al.*, 2002). Thus, rereplication may be another potential source for the genomic instability associated with tumorigenesis.

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Genome-wide Mapping of DNA Synthesis in *Saccharomyces cerevisiae* Reveals That Mechanisms Preventing Reinitiation of DNA Replication Are Not Redundant^D

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To maintain genomic stability, reinitiation of eukaryotic DNA replication within a single cell cycle is blocked by multiple mechanisms that inactivate or remove replication proteins after G1 phase. Consistent with the prevailing notion that these mechanisms are redundant, we previously showed that simultaneous deregulation of three replication proteins, ORC, Cdc6, and Mcm2-7, was necessary to cause detectable bulk re-replication in G2/M phase in *Saccharomyces cerevisiae*. In this study, we used microarray comparative genomic hybridization (CGH) to provide a more comprehensive and detailed analysis of re-replication. This genome-wide analysis suggests that reinitiation in G2/M phase primarily occurs at a subset of both active and latent origins, but is independent of chromosomal determinants that specify the use and timing of these origins in S phase. We demonstrate that re-replication can be induced within S phase, but differs in amount and location from re-replication in G2/M phase, illustrating the dynamic nature of DNA replication controls. Finally, we show that very limited re-replication can be detected by microarray CGH when only two replication proteins are deregulated, suggesting that the mechanisms blocking re-replication are not redundant. Therefore we propose that eukaryotic re-replication at levels below current detection limits may be more prevalent and a greater source of genomic instability than previously appreciated.

INTRODUCTION

Eukaryotic cells must replicate each portion of their genome precisely once per cell cycle to faithfully transmit that genome to succeeding generations. This cell cycle control is enforced at the hundreds to thousands of replication origins where replication is initiated. As part of this regulation, cells must prohibit reinitiation within a single cell cycle at every origin for many successive generations. Even a small or occasional slip in this control will lead to re-replication, which can potentially compromise genome integrity. Hence, the block to reinitiation must be absolutely effective and reliable.

Studies from many laboratories have led to a model for the block to reinitiation that is based on the division of the initiation event into two mutually exclusive stages (reviewed in Bell and Dutta, 2002; Diffley, 2004; Machida *et al.*, 2005). In the first stage, which is restricted to G1 phase, potential origins are selected on chromosomal DNA by assembly of the origin recognition complex (ORC), Cdc6, Cdt1, and the putative replicative helicase, Mcm2-7 into pre-replicative complexes (pre-RCs). In the second stage, which is restricted to S, G2, and M phases,

potential origins are activated to initiate DNA replication by two kinases, a cyclin-dependent kinase (CDK) and Cdc7 kinase. Because CDK activity prevents pre-RC assembly in S, G2, and M phases and origins are not activated in G1 phase, passage through the cell cycle is coupled to exactly one round of replication.

Although this model provides a framework for understanding once and only once initiation, it does not explain how the block to reinitiation can be maintained with such high fidelity. This fidelity can be readily incorporated into the model if multiple overlapping mechanisms prevent pre-RC reassembly. In fact, multiple CDK-dependent inhibitory mechanisms that target pre-RC components have been identified in a number of eukaryotic organisms. In budding and fission yeast, CDKs appear to down-regulate ORC through inhibitory phosphorylation of Orc2 and/or Orc6 (Nguyen *et al.*, 2001; Vas *et al.*, 2001) as well as by direct binding to Orc6 (Wilmes *et al.*, 2004). Additionally, CDKs inhibit Cdc6 (or the *Schizosaccharomyces pombe* ortholog Cdc18) by promoting Cdc6/Cdc18 degradation (Drury *et al.*, 1997, 2000; Jallepalli *et al.*, 1997; Elsasser *et al.*, 1999), by reducing CDC6 transcription (Moll *et al.*, 1991), and by directly inhibiting Cdc6/Cdc18 through phosphorylation (Jallepalli *et al.*, 1997) or binding (Mimura *et al.*, 2004). Finally, CDKs also promote the nuclear exclusion of Mcm2-7 and Cdt1 in budding yeast (Labib *et al.*, 1999; Nguyen *et al.*, 2000; Tanaka and Diffley, 2002), in part by direct phosphorylation of Mcm3 (Liku *et al.*, 2005). In metazoans, CDKs have been implicated in Orc1 degradation, Cdt1 degradation and Cdc6 nuclear exclusion (reviewed in Diffley, 2004). In addition, metazoan cells

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have a CDK-independent mechanism involving the protein geminin, which binds to Cdt1 and can prevent it from recruiting Mcm2-7 during S, G2, and M phase (reviewed in Blow and Dutta, 2005).

Obtaining clear evidence of re-replication within a single cell cycle has generally required the simultaneous disruption of multiple mechanisms, leading to the presumption that these mechanisms are redundant (Diffley, 2004; Blow and Dutta, 2005). In budding yeast, for example, simultaneous deregulation of ORC phosphorylation, Mcm localization, and Cdc6 protein levels was needed to detect re-replication in G2/M phase (Nguyen et al., 2001). Similarly, disruption of several regulatory mechanisms leads to re-replication in fission yeast (Gopalakrishnan et al., 2001; Vas et al., 2001; Yanow et al., 2001) and in *Xenopus* replication extracts (McGarry and Kirschner, 1998; Arias and Walter, 2005; Li and Blow, 2005; Yoshida et al., 2005).

In addition to the issue of mechanistic redundancy, the model for the block to re-replication makes predictions that are best examined by a genome-wide analysis of re-replication. First, the re-replication that is induced by deregulating pre-RC assembly should initiate from the potential replication origins used during normal replication. Reinitiation from a few origins has been observed by two-dimensional gel electrophoresis in both budding (Nguyen et al., 2001) and fission (Yanow et al., 2001) yeast, but genome-wide mapping of reinitiation sites is needed to confirm this prediction. Second, deregulation of pre-RC reassembly should be able to induce re-replication throughout the period from S to M phase. Although Cdt1 overexpression has been shown to prolong S phase in *Drosophila* embryos (Thomer et al., 2004), direct evidence for re-replication within S phase is still lacking. Finally, full deregulation of pre-RC reassembly should allow more than one round of reinitiation and result in rampant re-replication. So far, precise deregulation of replication proteins has led to at most a doubling of genomic DNA content, suggesting that additional inhibitory mechanisms remain to prevent re-replication. A more comprehensive analysis of where re-replication occurs in the genome may provide clues to how re-replication is still inhibited.

We have developed a more sensitive and comprehensive assay for re-replication by adapting and streamlining previously published microarray-based assays for analyzing DNA replication in budding yeast. With this assay we present evidence that reinitiation occurs primarily at a subset of the potential origins normally established for S phase without being strongly affected by the chromosomal determinants that specify the efficiency and timing of these origins in S phase. Our studies suggest that the limited re-replication observed may be due in part to the fewer initiation sites used for re-replication compared with S phase. Additionally, our studies indicate that some of the mechanisms preventing re-replication in G2/M phase also operate in S phase but that the block to re-replication in these two phases is not identical. Finally, we demonstrate that reinitiation from as few as a single origin is detectable when fewer mechanisms are disrupted, consistent with the notion that these mechanisms are not redundant but are each actively maintaining the high fidelity of the block to re-replication.

MATERIALS AND METHODS

Plasmids and Strains

All plasmids are described in Table 1, all strains are described in Table 2, and all oligonucleotides are described in Table 3. Supplementary Methods contains detailed description of plasmid and strain construction.

Table 1. Plasmids used in this study

Plasmid	Key features	Source
pJL737	ORC6 URA3	Nguyen et al. (2001)
pJL806	pGAL1 URA3	Nguyen et al. (2001)
pJL1206	MCM7-(SVNLS)2 URA3	Nguyen et al. (2001)
pJL1488	pGAL1-Δntcdc6-cdk2A URA3	This study
pJL1489	pGAL1-Δntcdc6 URA3	Nguyen et al. (2001)
pKI1260	MCM7-(svnls3A)2 URA3	Nguyen et al. (2001)
pMP933	ORC2 URA3	Nguyen et al. (2001)
YIp22	pMET3-HA3-CDC20 TRP1	Uhlmann et al. (2000)
pFA6a	KanMX6	Wach et al. (1994)
pAG25	NatMX4	Goldstein et al. (1999)
pPP117	cdc7-1 URA3	Hollingsworth et al. (1992)

Yeast Media, Growth, and Arrest

Cells were grown in YEP, synthetic complete (SC), or synthetic (S) broth medium (Guthrie and Fink, 1990) supplemented with 2% dextrose (wt/vol), 2% galactose (wt/vol), 3% raffinose (wt/vol), or 3% raffinose (wt/vol) + 0.05% dextrose (wt/vol). For S phase experiments cells were grown overnight in SDC (YJL5038) or SDC-Met,Ura (YJL3248 and YJL5834) and arrested in G1 phase with 50 ng/ml α factor (all strains were *bar1*) at 30°C. Cells were released by filtering, washing, and then resuspending in prewarmed 30°C YEPD containing 100 μ g/ml pronase, 100 mM hydroxyurea (HU), and 15 μ g/ml nocodazole.

To obtain reproducible induction of re-replication, cells were inoculated from a fresh unsaturated culture containing 2% dextrose into a culture containing 3% raffinose + 0.05% dextrose and grown for 12–15 h the night before the experiment. The GAL1 promoter (*pGAL1*) was induced by addition of 2% galactose and the MET3 promoter (*pMET3*) was repressed by the addition of 2 mM methionine. All experiments were performed at 30°C except where noted. For induction of re-replication in G2/M phase, cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were pelleted and resuspended in YEPRaff + 2 mM methionine and 15 μ g/ml nocodazole. Once arrested (>90% large budded cells), galactose was added to a final concentration of 2%. In experiments with strains containing *cdc7-1*, cells were grown and arrested at 23°C. These cultures were split after arresting in G2/M phase and either kept at 23°C or shifted to 35°C for 1 h followed by addition of 2% galactose to both cultures.

For induction of re-replication during the release from G1 phase into a G2/M phase arrest, cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were arrested with 50 ng/ml α factor (all strains were *bar1*). Once arrested (>95% small budded cells), galactose was added to a final concentration of 2% for 30 min. Cells were released by filtering, washing, and then resuspending in prewarmed YEPGal + 2 mM methionine, 100 μ g/ml pronase, and 15 μ g/ml nocodazole. For the induction of re-replication during a release from G1 phase into S phase, cells arrested and released as described above were resuspended in prewarmed YEPGal + 2 mM methionine, 100 μ g/ml pronase, and 100 mM HU.

Flow Cytometry

Cells were fixed and stained with 1 μ M Sytox Green (Molecular Probes, Eugene, OR) as previously described (Haase and Lew, 1997).

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as described in Green and Li (2005). Probes for ARS305, ARS607, and ARS1413 were prepared as described in Nguyen et al. (2001).

Two-Dimensional Gel Electrophoresis

Neutral-neutral two-dimensional (2-D) gel analysis was performed essentially as described at <http://fangman-brewer.genetics.washington.edu>. The DNA preparation described there is a slight modification of the one used in Huberman et al. (1987). Modifications to the previous protocols can be found in Supplementary Methods.

Microarray Assay

Microarrays containing 12,034 PCR products representing every ORF and intergenic region were prepared essentially as described (DeRisi et al., 1997; Iyer et al., 2001; see Supplementary Methods). Genomic DNA was prepared, labeled, and hybridized as described in Supplementary Methods.

Table 2. Strains used in this study

Strain	Genotype	Source
YJL310	<i>leu2-3,112 ura3-52 trp1-289 bar1Δ::LEU2</i>	Detweiler and Li (1998)
YJL3244	<i>orc2-cdk6A orc6-cdk4A leu2 ura3-52::[pGAL1, URA3] trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1]</i>	Nguyen <i>et al.</i> (2001)
YJL3248	<i>orc2-cdk6A orc6-cdk4A ura3-52::[pGAL1-Δntcdc6, URA3] trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1]</i>	Nguyen <i>et al.</i> (2001)
YJL3249	<i>orc2-cdk6A orc6-cdk4A ura3-52::[pGAL1-Δntcdc6, URA3] trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1]</i>	This study
YJL4486	<i>ORC2 ORC6 leu2 ura3-52::[pGAL1, URA3] trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1]</i>	This study
YJL4489	<i>ORC2 ORC6 ura3-52::[pGAL1-Δntcdc6-cdk2A, URA3] trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1]</i>	This study
YJL4832	<i>orc2-cdk6A orc6-cdk4A ura3-52::[pGAL1, URA3] trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1]</i>	This study
YJL3240	<i>orc2-cdk6A orc6-cdk4A ura3-52::[pGAL1-Δntcdc6, URA3] trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1]</i>	This study
YJL5038	<i>his3Δ::KanMX leu2Δ0 met15Δ0 ura3Δ0 bar1Δ::NatMX4 can1Δ::pMFA1-HIS3::pMFα1-LEU2</i>	This study
YJL5493	<i>orc2-cdk6A orc6-cdk4A leu2 ura3-52::[pGAL1, URA3] trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1]</i>	This study
YJL5834	<i>ORC2 ORC6 leu2 ura3-52::[pGAL1, URA3] trp1-289 ade2 ade3 MCM7 bar1Δ::LEU2</i>	This study
YJL5787	<i>ORC2 ORC6 ura3-52::[pGAL1-Δntcdc6-cdk2A, URA3] trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1] Δars316::KanMX6</i>	This study
YJL5858	<i>ORC2 ORC6 ura3-52::[pGAL1-Δntcdc6-cdk2A, URA3] trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1] Δars317::KanMX6</i>	This study
YJL5861	<i>ORC2 ORC6 ura3-52::[pGAL1-Δntcdc6-cdk2A, URA3] trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1] Δars318::KanMX4</i>	This study
YJL5816	<i>ORC2 ORC6 leu2 ura3-52::[pGAL1, URA3] trp1-289 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1] cdc7-1</i>	This study
YJL5822	<i>ORC2 ORC6 ura3-52::[pGAL1-Δntcdc6-cdk2A, URA3] trp1-289 leu2 ade2 ade3 MCM7-2NLS bar1Δ::LEU2 cdc20::[pMET3-HA3-CDC20, TRP1] cdc7-1</i>	This study

Data Analysis

Raw Cy5/Cy3 ratios from scanned arrays were normalized to the DNA content per cell based on the flow cytometry data to determine absolute copy number of each DNA segment. Raw values were then binned and smoothed using Fourier convolution smoothing essentially as described (Raghuraman *et al.*, 2001). Peaks in the replication profiles that were both prominent and reproducible among repetitions of an experiment were identified as origins. Details of data analysis (Supplementary Methods) and examples of raw data (Supplementary Figure S1) are contained in Supplementary Information. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE4181.

The "experiment variability" was determined using the equation for calculating one SD. Because there were only two DNA preparations used, each of which was hybridized twice, the trials are not truly independent and thus we call these values "experiment variability" rather than SD.

Scatter Plot

For each pro-ARS (Wyrick *et al.*, 2001), the normalized Cy5/Cy3 ratio of that chromosomal locus during replication or re-replication was determined and plotted. See Supplementary Methods for more details.

RESULTS

A Simplified Microarray CGH Assay for DNA Replication

We have adapted and streamlined existing microarray assays (Raghuraman *et al.*, 2001; Yabuki *et al.*, 2002) to create a rapid and economical genome-wide assay for yeast DNA replication. Our simplified assay uses CGH to directly measure the increase in DNA copy number arising from replication or re-replication. During S phase replication, the copy

Table 3. Oligonucleotides used in this study

Oligo	Purpose	Sequence
OJL1596	<i>ars316Δ</i>	5'-TTAACTGACAATTCTTTGAACAAAATTACACTTCATCAAGAAAGATGCCGGATCCCC GGGTTAATTAA-3'
OJL1597	<i>ars316Δ</i>	5'-TGATGACGAAGGATTCTGTGAAGTTGAATGCACACAAAAAAAGCTTGATACATCGATG AATTCTGAGCTCG-3'
OJL1639	<i>ars317Δ</i>	5'-ATTAACAAATGTTGATTTTAAATCGCAATTAAATACCCGGATCCCCGGGTTAATTAA-3'
OJL1640	<i>ars317Δ</i>	5'-ATTTTATGGAAGATTAAGCTCATAACTTGGACGGGGATCCATCGATGAATTGAGCTCG-3'
OJL1641	<i>ars318Δ</i>	5'-CGATAAAGTTATTATAGATTACATGTACCAACATTTGGATCCCCGGGTTAATTAA-3'
OJL1642	<i>ars318Δ</i>	5'-AGAGAAAATAGCTATTACCTAACATTAAAGGTATTAACATCGATGAATTGAGCTCG-3'
OJL1607	<i>ARS317 probe</i>	5'-ATCGATTATCTGTTGGCAGG-3'
OJL1608	<i>ARS317 probe</i>	5'-GAATTCAAAGAAGTCAATCTTATG-3'
OJL1452	<i>bar1Δ</i>	5'-ATTAAAAATGACTATATTTGATATTATGCTATAAGAAATTGTACTCCAGATTTC ATCGATGAATTGAGCTCG-3'
OJL1454	<i>bar1Δ</i>	5'-AGTGGTTCGTATCGCCTAAATCATAACCAAAATAGAGTGTCTAGAAGGGTCATA TACGGATCCCCGGGTTAATTAA-3'

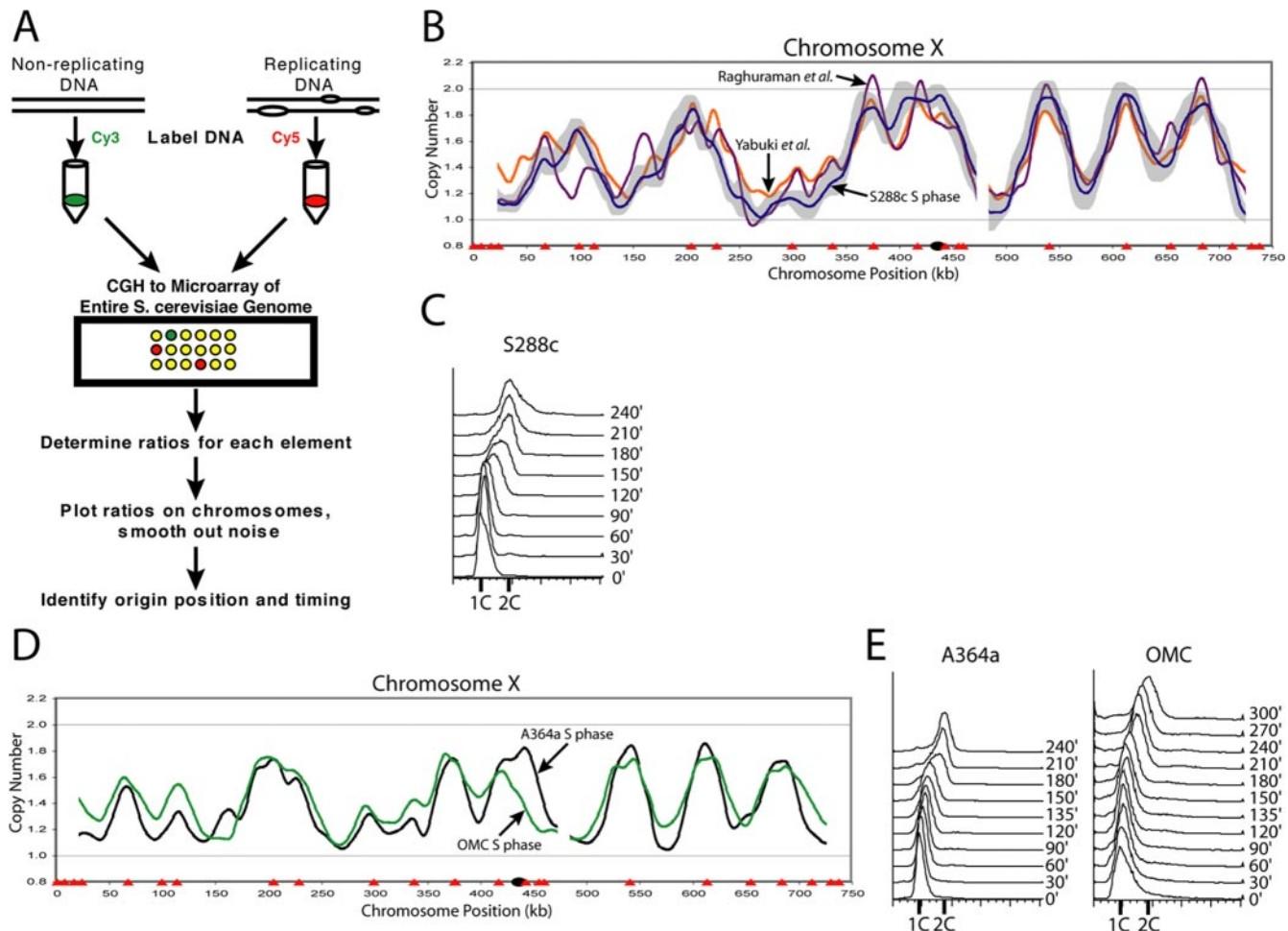


Figure 1. Use of comparative genomic hybridization (CGH) on spotted microarrays to assay DNA replication. (A) Schematic representation of the CGH replication assay. Genomic DNA is purified from nonreplicating and replicating cells, differentially labeled with Cy3 and Cy5, and competitively hybridized to a microarray containing 12,034 ORF and intergenic PCR products. Cy5/Cy3 ratios are normalized so that the average ratio of all elements equals the DNA content of the cells (as determined by flow cytometry). Normalized ratios are plotted against chromosomal position and mathematically smoothed to generate a replication profile. In most cases, two hybridizations are performed from each of two independent experiments. The resulting four replication profiles are averaged into one composite profile, and the locations of origins are identified using a peak finding algorithm. Chromosomal regions lacking data of sufficient quality are represented as gaps in the profiles. (B) CGH replication assay described for A was performed on YJL5038, a wild-type yeast strain in the S288c background. G1 phase genomic DNA was hybridized against S phase genomic DNA obtained 120 min after cells were released from G1 phase into media containing hydroxyurea (HU). The composite replication profile (blue line) plus and minus the “experiment variability” (light gray band; see Materials and Methods) is shown for chromosome X. Positions of origins annotated in the *Saccharomyces* Genome Database (SGD; Balakrishnan (2006); red triangles) and the centromere (black circle) are marked along the X-axis. Replication profiles derived from Raghuraman et al. (2001) (violet line) and Yabuki et al. (2002) (orange line) are shown for comparison. (C) S phase progression assayed by flow cytometry for experiment described in B at the indicated times after release from G1 phase. DNA content of 1.4 C was used to normalize the S288c replication profile. (D) The S phase replication profile of the re-replication competent OMC strain and the congenic wild-type strain are similar. S phase replication profiles were generated for the OMC strain YJL3248 (*MCM7-2NLS orc2-cdk6A orc6-cdk4A pGAL1-Δntcd6 pMET3-HA3-CDC20*) and a congenic wild-type A364a strain YJL5834 (*pGAL1*) essentially as described in B except S phase cells were harvested, respectively, at 135 min and 180 min after α factor release. The S phase replication profile for the OMC strain (green line) and the A364a strain (black line) for chromosome X is shown. SGD annotated origins (red triangles) and the centromere (black circle) are marked along the X-axis. (E) S phase progression assayed by flow cytometry for experiment described in D at the indicated times after release from G1 phase. DNA contents of 1.35 C and 1.4 C, respectively, were used to normalize the OMC and A364a replication profiles.

number of each DNA segment reflects the timing of its replication because the earlier a DNA segment replicates, the greater the proportion of replicating cells containing a duplication of this segment. Origins, which replicate earlier than neighboring regions, can be localized to chromosomal segments where the copy number reaches a local maxima. Thus, use of microarray CGH to monitor copy number changes across the genome can provide a comprehensive

view of the location and efficiency/timing of initiation sites during replication and re-replication.

Figure 1A shows a schematic of our microarray CGH replication assay. Genomic DNA from replicating (or re-replicating) and nonreplicating cells is purified and differentially labeled with Cy5 and Cy3. The labeled probes are competitively hybridized to a spotted microarray and the raw Cy5/Cy3 values are normalized such that the average

ratio corresponds to the DNA content determined by flow cytometry. Data are smoothed and origins are computationally identified by locating prominent and reproducible peaks in smoothed replication profiles.

Before using the microarray CGH assay to study re-replication, we assessed its reproducibility and its ability to identify known replication origins in the S phase of a wild-type S288c strain (flow cytometry data in Figure 1C). Figure 1B and Supplementary Figure S2 show the mean of the smoothed S phase replication profiles from four hybridizations plus or minus the "experiment variability" (see *Materials and Methods*) for chromosome X. The small variability demonstrates that this technique is highly reproducible. An overlay of our replication profiles with those generated from previously published data (Raghuraman *et al.*, 2001; Yabuki *et al.*, 2002) shows considerable agreement in both peak positions, which reflects origin locations, and peak heights, which reflects origin timing/efficiency. When our peak finding algorithm was applied to our profiles, we obtained origin numbers (212) comparable to those obtained by Rhaguraman *et al.* (2001) (332) and Yabuki *et al.* (2002) (260). Additionally, the alignment of peaks to origins systematically mapped by 2-D gel electrophoresis or ARS plasmid assay was similar to, or better than, published data (Supplementary Table S1). Together, these data confirm that our streamlined assay is reproducible and accurate.

Re-replication Competent Mutant Has a Mostly Normal S Phase

We have previously demonstrated that simultaneous deregulation of three pre-RC components (ORC, Mcm2-7, and Cdc6) leads to limited re-replication in G2/M phase arrested cells (Nguyen *et al.*, 2001). These initiation proteins were deregulated by mutations that make the proteins refractory to CDK regulation. First, the CDK consensus phosphorylation sites of two subunits of the origin recognition complex, Orc2 and Orc6, were mutated, preventing Cdc28/Cdk1 phosphorylation of these subunits (*orc2-cdk6A*, *orc6-cdk4A*). Second, two copies of the SV40 nuclear localization signal were fused to *MCM7* (*MCM7-SVNL_S*) to prevent the Cdc28/Cdk1 promoted net nuclear export of the Mcm2-7 complexes. Finally, an extra copy of *CDC6*, containing a partially stabilizing N-terminal deletion, was placed under control of the galactose inducible promoter (*pGAL1-Δntcdc6*). This strain re-replicates when *Δntcdc6* is induced by addition of galactose and will be referred to as the OMC re-replicating strain in reference to its deregulation of ORC, Mcm2-7, and Cdc6.

A major concern in any genetic analysis of replication control is the possibility that the mutations deregulating replication proteins also disrupt their replication activity. Such a nonspecific perturbation would complicate any interpretation of the resulting phenotype. We and others have previously reported that *Δntcdc6* expressed under the *CDC6* promoter retains full replication initiation function (Drury *et al.*, 2000; Nguyen *et al.*, 2001). To determine whether the mutations deregulating Orc2, Orc6, and Mcm7 in the OMC strain also preserve their initiation function, we compared S phase of the OMC strain (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6*), when re-replication was not induced, to S phase of the congenic wild-type A364a strain (*ORC2 ORC6 MCM7 pGAL1*). When cells were harvested at the same point in S phase (Figure 1E), the replication profiles for the two strains showed considerable overlap (Figure 1D, Supplementary Figures S3 and S4), although ORC and Mcm7 mutations cause subtle alterations in the initiation of DNA replication. Because two wild-type strains of different strain backgrounds show nearly identical replication profiles (Supplementary Figures S5 and S6), we believe these differ-

ences reflect subtle alterations in the initiation activity of the mutant ORC and Mcm2-7. Nonetheless, we conclude that, overall, the mutant ORC and Mcm2-7 proteins in the OMC strain retain most of their normal initiation activity.

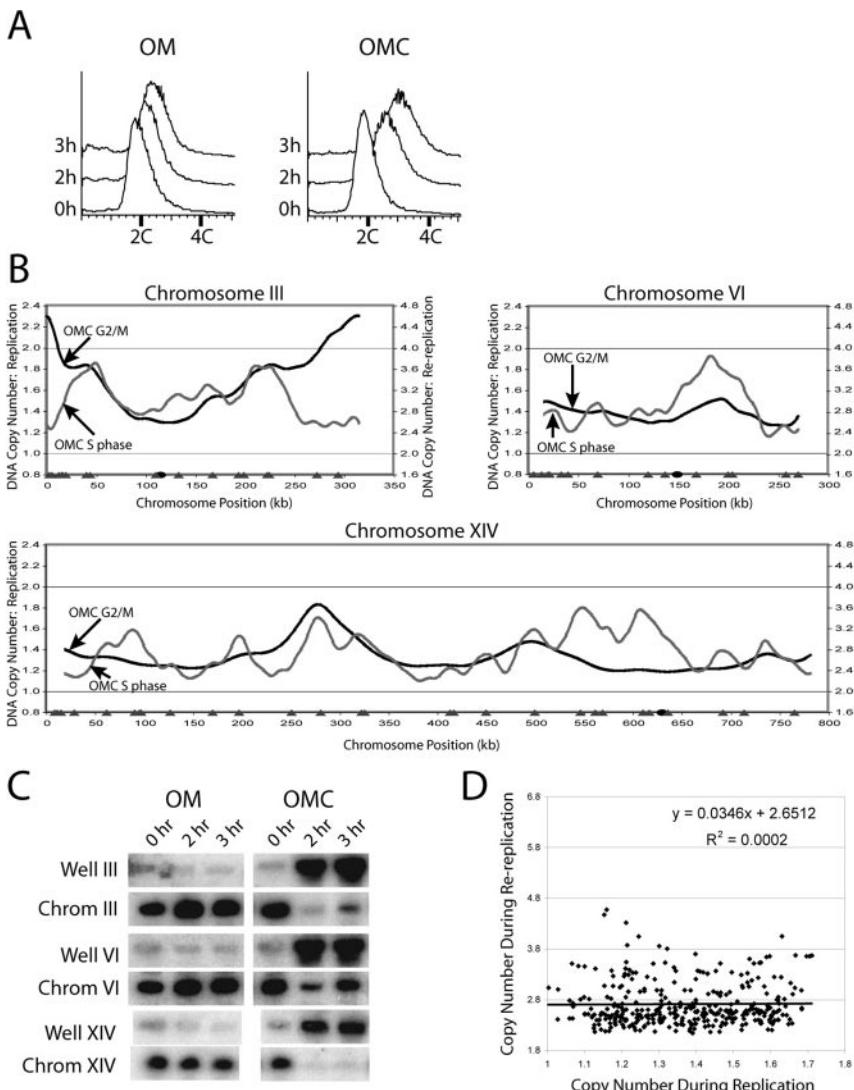
Mapping Reinitiating Origins

A key prediction of the current model for eukaryotic replication control is that pre-RC reassembly and reinitiation should only occur where pre-RCs normally assemble, i.e., the potential origins or pro-ARSs identified by Wyrick *et al.* (2001). In our previous characterization of re-replication induced at G2/M phase in the OMC strain (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6*), we observed three active S phase origins reinitiating by 2-D gel electrophoresis (Nguyen *et al.*, 2001). To comprehensively examine this prediction throughout the genome, we performed microarray CGH on the re-replicating DNA from OMC cells. This re-replicating DNA (flow cytometry in Figure 2A) was competitively hybridized against DNA from a congenic non-re-replicating strain that lacks the inducible *Δntcdc6* and will be referred to as the OM strain (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1*). Another source of non-re-replicating control DNA is OMC DNA from G1 phase cells, and when this was used, virtually identical results were obtained (unpublished data).

The OMC G2/M phase re-replication profiles are shown in Figure 2B and Supplementary Figure S7. These data confirm that the incomplete re-replication observed by flow cytometry is distributed over all 16 chromosomes, as was first suggested by their limited entry into the gel during PFGE (Nguyen *et al.*, 2001 and Figure 2C). The re-replication profiles also show that individual chromosomes re-replicate very unevenly, with some segments preferentially re-replicating more than others do.

Application of a peak finding algorithm to OMC re-replication profiles identified 106 reinitiating origins. Most of these origins appear to correspond to chromosomal loci that form pre-RCs in G1 phase because more than 80% of the reinitiating origins map to within 10 kb of a pro-ARS identified by Wyrick *et al.* (2001) as sites of pre-RC binding. The mean distance between the OMC reinitiating origins and the closest Wyrick pro-ARS (Wyrick *et al.*, 2001) is 7.0 kb. This value is highly significant ($p < 5 \times 10^{-8}$) when compared with the mean distances calculated for equivalent numbers of randomly selected chromosomal loci, as the mean distances are tightly distributed around a value of 12.3 kb (Supplementary Figure S8).

Tanny *et al.* (2006) have analyzed the re-replication profile of a strain similar to our OMC strain containing the additional perturbation of a mutation of an RXL motif in ORC6 that abrogates CDK binding and results in a slightly increased extent of re-replication. Although both articles use slightly different data analysis and presentation, (our profiles are presented to preserve absolute copy number information at the cost of less distinctive peaks), the re-replication profiles are strikingly similar (compare Supplementary Figure S7 to Tanny *et al.*, 2006; Supplementary Figure S2). Like our results, 80% of the 123 re-replication origins identified by Tanny *et al.* (2006) are within 10 kb of a Wyrick *et al.* (2001) pro-ARS, further supporting the notion that re-replication occurs at normal sites of pre-RC formation. Overlap of origins identified in both studies is considerable, with 64% of the origins in this study within 10 kb of an origin in Tanny *et al.* (2006) (20% would be expected by chance). This overlap becomes even more striking, 80% overlap (expected value is also 20%), when the top 40 highest peaks in our analysis are compared with peaks identified in Tanny *et al.* (2006). Together with our previous confirmation by 2-D gel



versus the DNA copy number from the OMC S phase replication profile in B. Line represents linear regression of plot.

electrophoresis that ARS305, ARS121, and ARS607 reinitiate (Nguyen et al., 2001), these genomic data suggest that reinitiation primarily occurs at a subset of potential S phase origins.

The efficiency with which these potential origins reinitiate in G2/M phase, however, does not correlate with the efficiency or timing with which they initiate in S phase. For example, only 38% of the active S phase origins reinitiate with enough efficiency to be identified as peaks during re-replication in G2/M phase. Moreover, some regions that normally replicate late in S phase, such as those near the telomeres of chromosome III, re-replicate very efficiently in G2/M phase, apparently from very inefficient or latent S phase origins in those regions. For a systematic comparison of re-replication efficiency versus replication timing of all potential S phase origins, we plotted the re-replication copy number versus the replication copy number for the set of pro-ARSs identified by Wyrick et al. (2001) (Figure 2D). The absence of any significant correlation ($R^2 = 0.0002$) indicates that the efficiency or timing of a replication origin in S phase does not determine its re-replication efficiency during G2/M phase.

Figure 2. Re-replication induced during G2/M phase when ORC, Mcm2-7, and Cdc6 are deregulated. (A) G2/M phase re-replication in the OMC strain is readily detectable by flow cytometry. The OMC strain YJL3248 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6 pMET3-HA3-CDC20*) and the control OM strain YJL3244 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) were arrested in G2/M phase. Once arrested, galactose was added, which induced re-replication in the OMC strain. Samples were taken for flow cytometry at the indicated points after galactose addition. The DNA content of 2.7 C at 3 h was used to normalize the OMC re-replication profile in B. (B) Genomic DNA was purified from the OMC strain and the control OM strain after 3 h of galactose induction as described in A and competitively hybridized against each other as described in Figure 1A. The OMC G2/M phase re-replication profiles (black lines, right axis), the OMC S phase replication profiles replotted from Figure 1D (gray lines, left axis), locations of pro-ARSs mapped by Wyrick et al. (2001) (gray triangles) and the centromeres (black circles) are shown for chromosomes III, VI, and XIV. (C) Each chromosome participates when OMC cells are induced to re-replicate in G2/M phase. The OMC strain and the control OM strain from the experiment presented in A were harvested for pulsed-field gel electrophoresis (PFGE) at the indicated times. Southern blots of the gel were probed with fragments containing ARS305 to detect chromosome III, ARS607 to detect chromosome VI, and ARS1413 to detect chromosome XIV. For each chromosome the Southern signal for both the gel well and the normal chromosomal position are shown. (D) Replication timing does not correlate with efficiency of G2/M phase re-replication in the OMC strain. For each of the pro-ARSs defined by Wyrick et al. (2001), the DNA copy number from the OMC G2/M phase re-replication profile in B was plotted

Mechanisms That Prevent Re-replication at G2/M Phase Also Act in S Phase

The prevailing model for replication control depicts the prevention of re-replication in S, G2, and M phase as one continuous inhibitory period using a common strategy of preventing pre-RC reassembly. Because CDKs are active throughout this period, the model would predict that mechanisms used by CDKs to regulate replication proteins should prevent re-replication throughout S, G2, and M phase. To determine if CDK regulation of ORC, Mcm2-7, and Cdc6, which prevents re-replication within G2/M phase, also prevents re-replication in S phase, we induced Δ ntcdc6 in OMC cells (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6*) as they entered S phase.

OMC cells were arrested in G1 phase with α factor, and half the cells were harvested to obtain G1 phase DNA. The remaining cells were induced to express Δ ntcdc6 and then released from the G1 arrest into a low concentration of HU to delay their replication and allow us to collect them in S phase. Flow cytometry indicated that the released cells were harvested while still in S phase with a DNA content of 1.4 C

(Figure 3A). The S phase and G1 phase DNA were competitively hybridized against the yeast genomic microarray to generate a combined replication/re-replication profile for S phase (Figure 3B and Supplementary Figure S9).

Because normal S phase replication can account for an increase in DNA copy number from 1 to 2, only DNA synthesis beyond this copy number can be unequivocally attributed to re-replication. As seen in Figure 3B and Supplementary Figure S9, many early origins acquired a DNA copy number greater than 2; in some cases reaching values greater than 3. In the same profiles other chromosomal regions had copy numbers significantly below 2, confirming that cells were indeed in the midst of S phase. In fact, early origins reinitiated, whereas forks from their first round of replication were still progressing and before many late origins had fired. Similar re-replication profiles were observed for re-replicating cells synchronously harvested in S phase in the absence of HU (unpublished data). These findings thus directly establish that mechanisms used to prevent re-replication in G2/M phase also act within S phase.

Cell Cycle Position Can Affect the Extent and Location of Re-replication

To determine if the block to re-replication is modulated during progression through the cell cycle, we compared the re-replication profile of OMC cells (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6*) that were induced to re-replicate through a complete S phase with the profile associated with re-replication in G2/M phase. To obtain the former profile, both OMC and control OM cells (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1*) were arrested in G1 phase with α factor followed by addition of galactose to induce Δ ntcdc6 in the OMC strain. Cells were then released from the G1 arrest, allowed to proceed through S phase, and collected at a G2/M arrest 3 h after the release. DNA prepared from the OMC and OM strains were competitively hybridized to our yeast genomic microarray to obtain a "G1 release" re-replication profile for the OMC cells.

Flow cytometry showed that both the re-replicating OMC and the control OM strain were in the middle of S phase 1 h after the release (Figure 4A). As expected for actively replicating chromosomes (Hennessy and Botstein, 1991), the chromosomes of these strains were retained in the wells during PFGE (Figure 4B). Two hours after the release, S phase was mostly complete in the control OM strain and its chromosomes reen-

tered the gel during PFGE. In the OMC strain, however, the induction of re-replication prevented chromosomes from reentering the PFGE gel at both 2 and 3 h time points. Because significant re-replication could be induced in OMC cells delayed in S phase, we believe that re-replication during the progression through S phase contributed to the re-replication seen in the G1 release experiment.

Re-replication induced during G1 release of OMC cells was more extensive than re-replication induced in G2/M phase. Despite comparable lengths of induction, flow cytometry reproducibly indicated that the former accumulated a DNA content of 3.2 C, whereas the latter accumulated only 2.7 C (compare 3 h time points in Figure 4A with Figure 2A). More extensive re-replication could also be seen by comparing the re-replication profiles induced during the G1 release (Figure 4C and Supplementary Figure S10) and the G2/M phase arrest (Figure 2B and Supplementary Figure S7). In general the peaks in the G1 release profiles were taller than the G2/M phase profiles, suggesting that more efficient or more rounds of reinitiation can occur when re-replication is induced during S phase. For example, *ARS305* reached a copy number of 6.6, indicating it reinitiated a second time, as a single round can only generate a maximum copy number of 4. Overall, multiple rounds of reinitiation were observed on more than half of the chromosomes when re-replication was induced during the G1 release. In contrast, multiple rounds of reinitiation occurred at much fewer loci and to a lesser extent when re-replication was induced in G2/M phase.

A peak finding algorithm identified 87 potential reinitiation sites when re-replication was induced during the G1 release experiment. Of these, 85% were located within 10 kb of a Wyrick pro-ARS Wyrick et al. (2001). These data suggest that re-replication induced during a G1 release occurs from S phase origins of DNA replication.

In addition to the extent of re-replication, another significant difference between re-replication induced during the G1 release and re-replication induced during G2/M phase was their pattern of origin usage. As discussed above, efficiency of re-replication in G2/M phase was not correlated with origin usage during S phase. In contrast, the efficiency of re-replication induced during the G1 release exhibited a modest positive correlation with S phase origin timing (Figure 4D). Although we cannot rule out an intrinsic difference in the reinitiation efficiency of early versus late origins when re-replication is induced during the G1 release, the simplest explanation for this

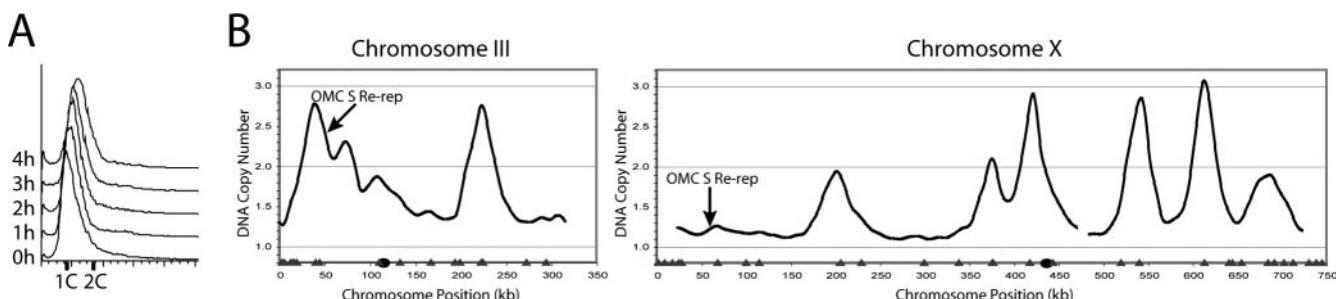


Figure 3. Deregulation of ORC, Mcm2-7, and Cdc6 can induce re-replication in S phase. (A) Flow cytometry of OMC cells induced to re-replicate in S phase. The OMC strain YJL3249 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6 pMET3-HA3-CDC20*) was arrested in G1 phase, induced to express Δ ntcdc6 by the addition of galactose, then released from the arrest into media containing HU to delay cells from exiting S phase. At 4 h the cells were still in S phase with a DNA content of 1.4 C. This value was used to normalize the re-replication profile in B. (B) OMC cells can reinitiate and re-replicate within S phase. Genomic DNA was isolated at the 0 h (G1 phase) and 4 h (S phase) time points from the OMC strain YJL3249 as described in A and competitively hybridized against each other. The resulting profiles shown for chromosomes III and X reflect copy number increases due to both replication and re-replication. Locations of pro-ARSs mapped by Wyrick et al. (2001) (gray triangles) and the centromeres (black circles) are plotted along the X-axis.

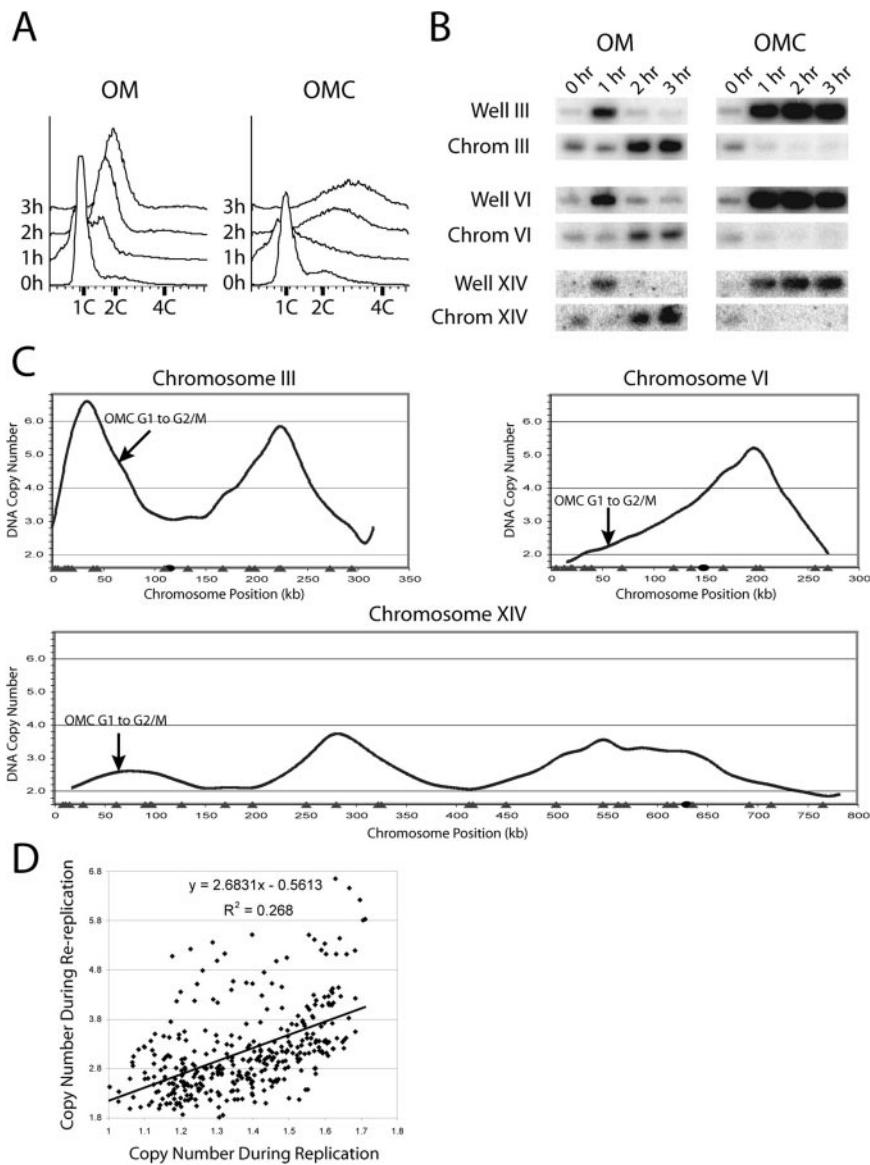


Figure 4. Re-replication induced upon release from a G1 arrest when ORC, Mcm2-7, and Cdc6 are deregulated. (A) Robust re-replication of OMC cells after G1 release. The OMC strain YJL3248 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6 pMET3-HA3-CDC20*) and the control OM strain YJL3244 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) were arrested in G1 phase, exposed to galactose to induce Δntcdc6 in the OMC strain, and then released from the arrest into G2/M phase. Samples were taken for flow cytometry at the indicated times after release from the α factor arrest. The OMC re-replication profile in C was normalized to the 3 h DNA content of 3.2. C. (B) Cells that were induced to re-replicate in A were harvested for PFGE at the indicated times. Southern blots of the gel were probed for chromosomes III, VI, and XIV as described in Figure 2C. (C) Re-replication profile of the OMC strain after G1 release. Genomic DNA was purified from the OMC strain and the control OM strain 3 h after G1 release. The two DNA preparations were labeled and competitively hybridized against each other to generate the G1 release re-replication profiles shown for chromosomes III, VI, and XIV. Locations of pro-ARSS mapped by Wyrick *et al.* (2001) (gray triangles) and the centromeres (black circles) are plotted along the X-axis. (D) Re-replication induced in the OMC strain after a G1 release is slightly biased toward early replicating pro-ARSS. For each of the pro-ARSS defined by Wyrick *et al.* (2001), the DNA copy number from the OMC G1 release re-replication profile in C was plotted versus the DNA copy number from the OMC S phase replication profile in Figure 2B. Line represents linear regression of plotted points.

correlation is that earlier replicating origins are cleared of pre-RCs earlier, making them available sooner for reassembly of pre-RCs and reinitiation within S phase.

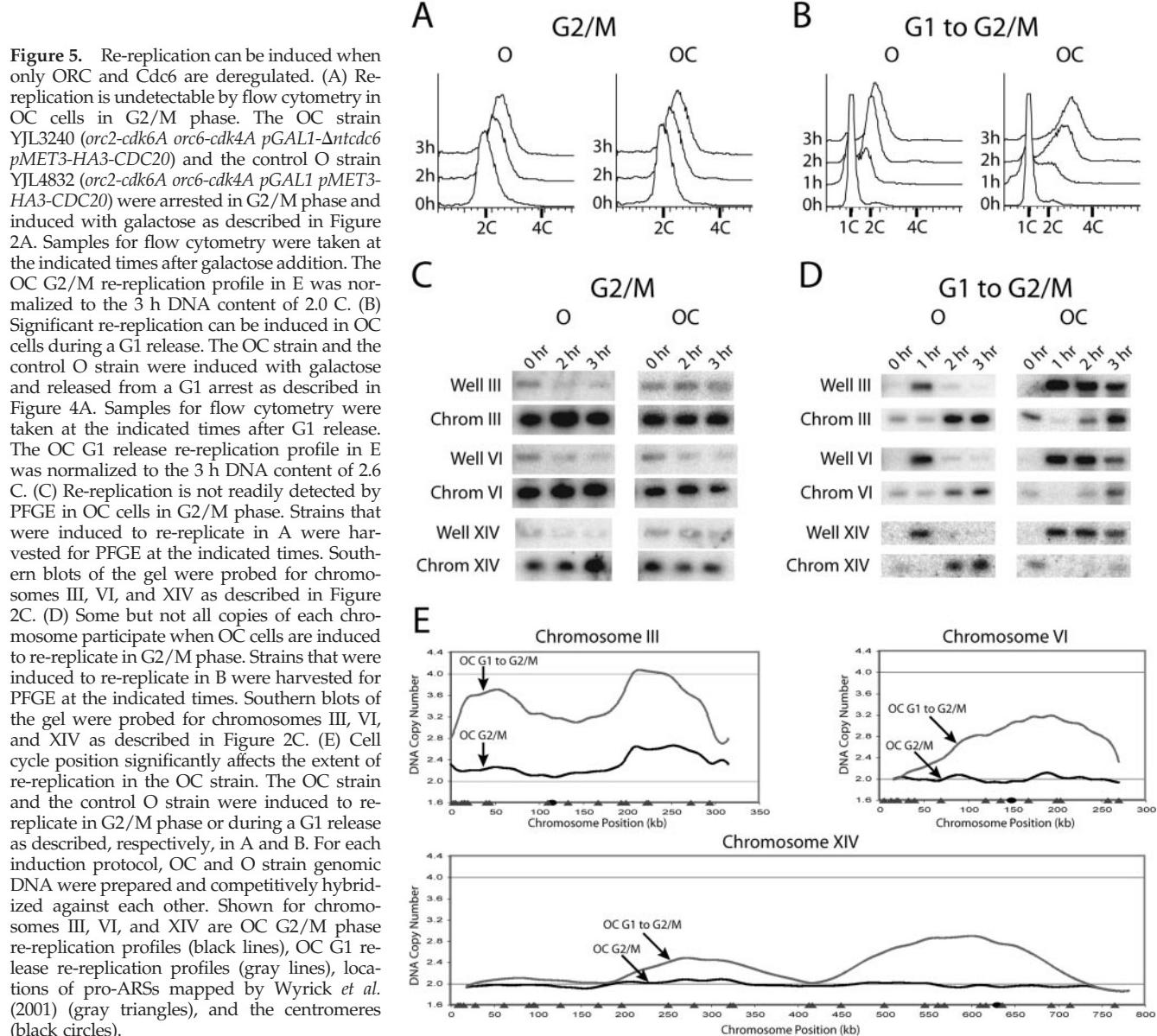
Limited Re-replication Is Detectable with Fewer Genetic Perturbations

Our previous analysis of budding yeast re-replication failed to detect re-replication when only two pre-RC components were deregulated in G2/M phase (Nguyen *et al.*, 2001). This observation is frequently cited as evidence that eukaryotic replication controls are highly redundant. Both the increased sensitivity of the microarray CGH assay and the enhanced re-replication observed during a G1 release provided opportunities to reexamine whether these controls are indeed redundant in budding yeast.

As a first step, we examined an “OC” strain (*orc2-cdk6A orc6-cdk4A pGAL1-Δntcdc6*), in which only ORC and Cdc6 are deregulated and compared it with a control “O” strain (*orc2-cdk6A orc6-cdk4A GAL1*), where only ORC is deregulated. In accordance with our previous results (Nguyen *et al.*,

2001), induction of Δntcdc6 in G2/M phase generated no significant increase in DNA content by flow cytometry (Figure 5A) or chromosome immobilization during PFGE (Figure 5C). Similarly, microarray CGH of DNA prepared from the OC and O strains after 3 h of galactose induction in G2/M phase detected no re-replication on 15 out of 16 chromosomes (Supplementary Figure S11). However, limited re-replication could clearly be observed on both arms of chromosome III (Figure 5E). Thus, the microarray CGH assay can detect re-replication missed by other assays.

We next asked whether we could detect more re-replication in the OC strain by inducing it during a G1 release. In contrast to the results obtained during a G2/M phase induction, significant re-replication was detected by flow cytometry and PFGE within 2 h of the G1 release (Figure 5, B and D). The re-replication profile of the OC strain induced during a G1 release (Figure 5E and Supplementary Figure S11) showed broad re-replication zones of ~200–500 kb in width on all chromosomes. These results, along with the re-replication induced during G2/M phase, establish that deregulation



lating just ORC and Cdc6 is sufficient to induce re-replication and thus these inhibitory mechanisms are not truly redundant. The greater amount of re-replication induced during G1 release versus G2/M arrest underscores the dynamic character of the block to re-replication and, in this case, is likely due to the incomplete expulsion of Mcm proteins from the nucleus during S phase.

Microarray CGH Can Detect Re-replication Initiating Primarily from a Single Origin

To further investigate the question of redundancy in replication control, we examined the consequences of deregulating just Mcm2-7 and Cdc6. We were not able to detect re-replication in the "MC" strain (*MCM7-2NLS pGAL1-Δntcdc6*) whether Δntcdc6 was induced in G2/M phase or during a G1 release (unpublished data). Hence, we further deregulated Cdc6 inhibition by mutating the two full CDK consensus phosphorylation sites on Δntcdc6 to generate the MC_{2A} strain (*MCM7-2NLS*

Δntcdc6-cdk2A). These additional mutations increase the stability of Δntcdc6 (Perkins *et al.*, 2001).

Expression of Δntcdc6-cdk2A in the MC_{2A} strain in either G2/M phase or during a G1 release did not cause a detectable increase in DNA content by flow cytometry (Figures 6, A and B). However, PFGE suggested that chromosome III re-replicated in a small subset of MC_{2A} cells when Δntcdc6-cdk2A was induced under either protocol (Figure 6, C and D). Microarray CGH provided definitive evidence that re-replication occurred, in this strain, primarily on the right arm of chromosome III (Figure 6E and Supplementary Figure S12).

To confirm that the very limited DNA re-replication in the MC_{2A} strain arose from a canonical reinitiation event, we asked whether this re-replication depended on known origins and initiation proteins. Our peak finding algorithm implicated an initiation event at ~297 kb, close to *ARS317*, an inefficient S phase origin located at 291 kb. Two-dimensional gel analysis of *ARS317* (Figure 7A) detected bubble

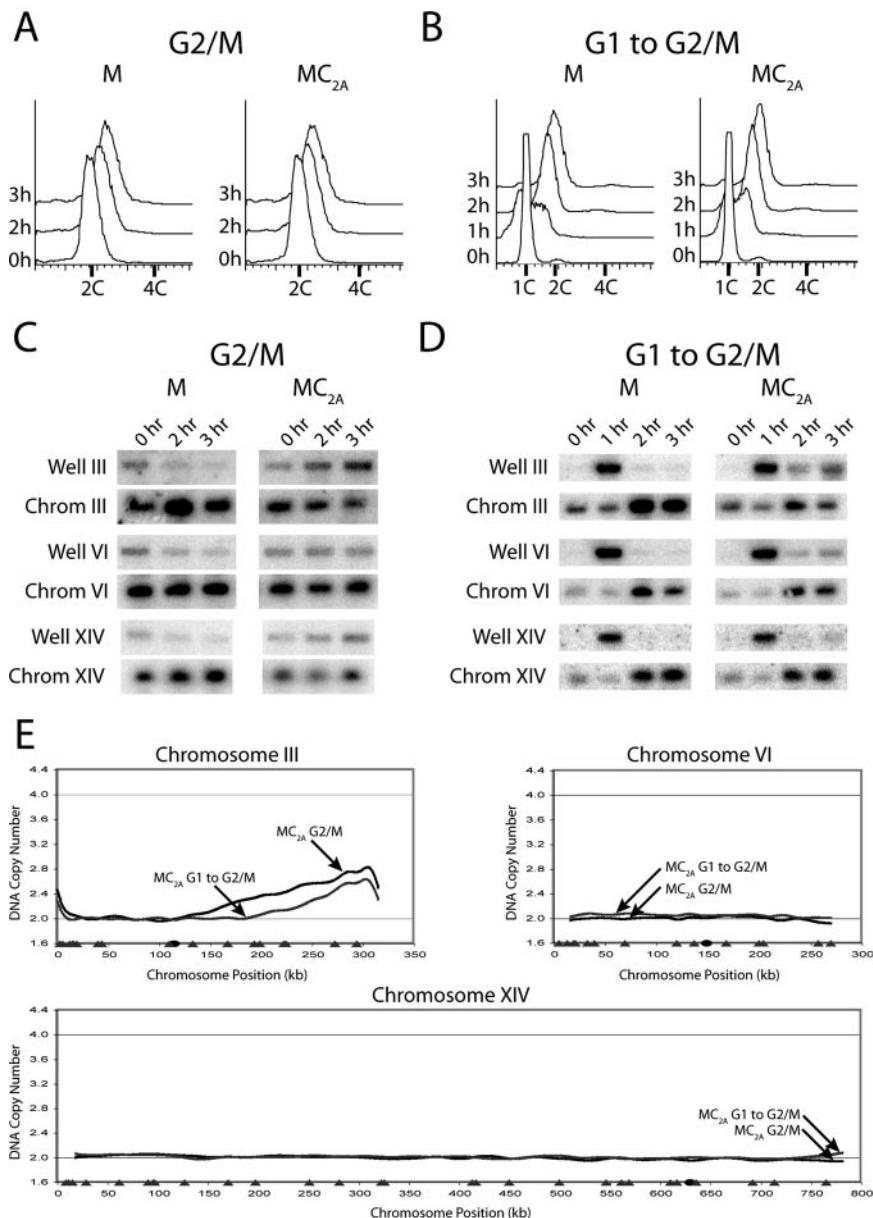


Figure 6. Re-replication occurs primarily on a single chromosome when Mcm2-7 and Cdc6 are deregulated. (A) Re-replication is undetectable by flow cytometry in MC_{2A} cells in G2/M phase. The MC_{2A} strain YJL4489 (*MCM7-NLS pGAL1-Δntcdc6-cdk2A pMET3-HA3-CDC20*) and the control M strain YJL4486 (*MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) were arrested in G2/M phase and induced with galactose as described in Figure 2A. Samples for flow cytometry were taken at the indicated times after galactose addition. The MC_{2A} G2/M re-replication profile in E was normalized to the 3 h DNA content of 2.0 C. (B) Re-replication is undetectable by flow cytometry in MC_{2A} cells during a G1 release. The MC_{2A} strain and the control M strain were induced with galactose and released from a G1 arrest as described in Figure 4A. Samples for flow cytometry were taken at the indicated times. The MC_{2A} G1 release re-replication profile in E was normalized to the 3 h DNA content of 2.0 C. (C) A portion of the population of chromosome III molecules participate when MC_{2A} cells are induced to re-replicate in G2/M phase. The strains that were induced to re-replicate in A were harvested for PFGE at the indicated times. Southern blots of the gel were probed for chromosomes III, VI, and XIV as described in Figure 2C. (D) A portion of the population of chromosome III molecules participate when MC_{2A} cells are induced to re-replicate during a G1 release. The strains that were induced to re-replicate in B were harvested for PFGE at the indicated times. Southern blots of the gel were probed for chromosomes III, VI, and XIV as described in Figure 2C. (E) Re-replication in the MC_{2A} strain occurs primarily on chromosome III. The MC_{2A} strain and the control M strain were induced to re-replicate in G2/M phase or during a G1 release as described, respectively, in A and B. For each induction protocol, MC_{2A} and M strain genomic DNA were prepared and competitively hybridized against each other. Shown for chromosomes III, VI, and XIV are MC_{2A} G2/M phase re-replication profiles (black lines), MC_{2A} G1 release re-replication profiles (gray lines) locations of pro-ARSs mapped by Wyrick *et al.* (2001) (gray triangles) and the centromeres (black circles).

arcs, indicative of replication initiation, in the MC_{2A} strain but not the control "M" strain (*MCM7-2NLS pGAL1*). The immediately adjacent origins, ARS316 and ARS318, only displayed fork arcs (unpublished data), suggesting that most of the re-replication on the right arm of chromosome III originates from ARS317. Deletion of ARS317, but not ARS316 or ARS318, in the MC_{2A} strain eliminated the bulk of the re-replication detected by microarray CGH (Figure 7B and unpublished data), demonstrating that re-replication initiates primarily from a single S phase origin.

We next asked whether this re-replication is dependent on the essential initiation factor, Cdc7-Dbf4 kinase. Both MC_{2A} and MC_{2A} *cdc7-1* strains were induced to re-replicate in G2/M phase under permissive (23°C) and restrictive (35°C) temperatures for the *cdc7-1* allele. Microarray CGH demonstrated that both strains re-replicated to a similar extent at 23°C (Supplementary Figure S13), but at 35°C there was little or no re-replication in the MC_{2A} *cdc7-1* strain (Figure 7C).

Together, the dependence on both ARS317 and Cdc7-Dbf4 indicates that the very limited re-replication induced in the MC_{2A} strain arises primarily from a single bona fide reinitiation event.

DISCUSSION

Use of Microarray CGH as a Routine Genome-wide Assay for Budding Yeast Replication

We have refined previously published genome-wide replication assays for budding yeast and made them more amenable for routine and widespread use in the study of eukaryotic DNA replication. The previous assays required significant effort and cost to generate a single replication profile and were only used to characterize the normal wild-type S phase (Raghuraman *et al.*, 2001; Yabuki *et al.*, 2002). We have obtained comparable replication profiles using a streamlined protocol,

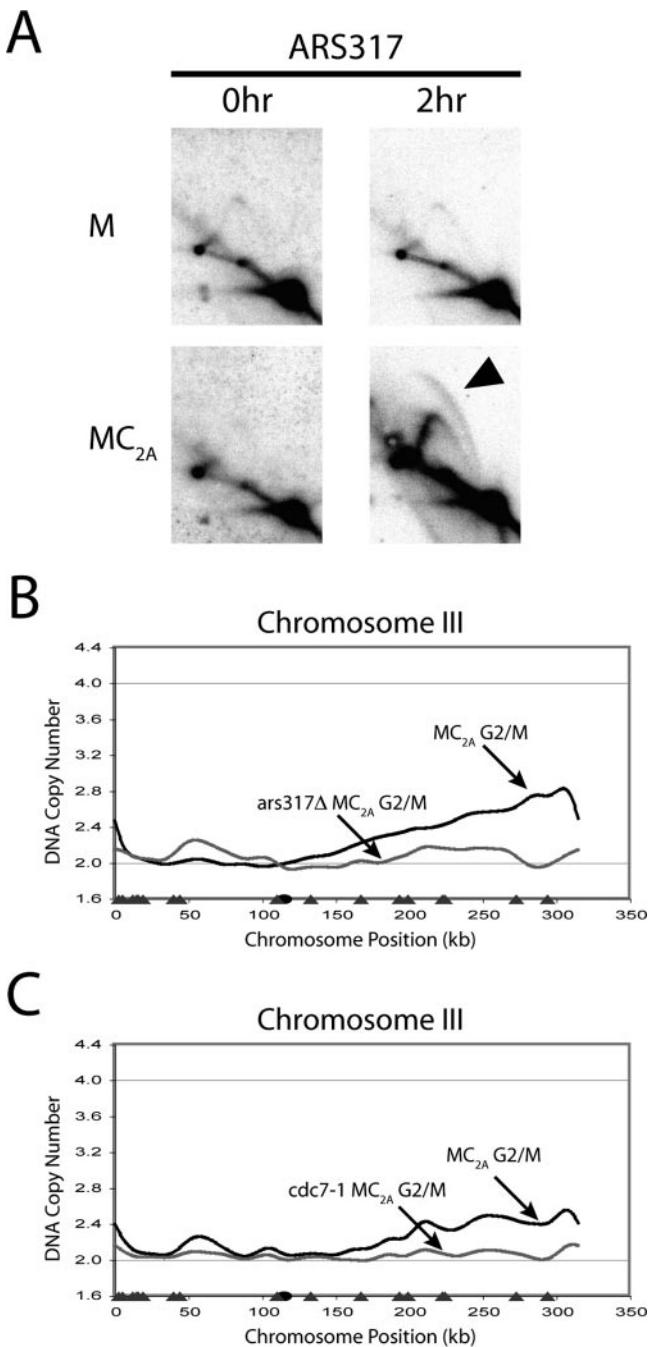


Figure 7. The re-replication arising from deregulation of both Mcm2-7 and Cdc6 depends on *ARS317* and *Cdc7*. (A) Reinitiation bubbles are induced at *ARS317* when *MC_{2A}* re-replicates in G2/M phase. The *MC_{2A}* strain YJL4489 (*MCM7-NLS pGAL1-Δntcd6-cdk2A pMET3-HA3-CDC20*) and the control M strain YJL4486 (*MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) were arrested in G2/M phase and induced with galactose as described in Figure 6A. Genomic DNA was purified from each strain at both 0 and 2 h after induction and subjected to neutral-neutral 2-D gel electrophoresis. Southern blots of the gels were probed with an *ARS317* fragment. Black arrow indicates re-replication bubbles. (B) *ARS317* sequence is required for the bulk of re-replication induced in *MC_{2A}* cells. The *MC_{2A}-Δars317* strain YJL5858 (*MCM7-NLS pGAL1-Δntcd6-cdk2A pMET3-HA3-CDC20 Δars317*) and the control M strain YJL4486 were arrested in G2/M phase and induced with galactose for 3 h as described in Figure 6A. Genomic DNA from the two strains was competitively hybridized against each other to generate the *MC_{2A}-Δars317* G2/M

collection of a single time point and inexpensive spotted microarrays. Thus, it is feasible to use our streamlined assay to examine the genome-wide replication phenotypes associated with many different genotypes or physiological conditions.

Reinitiation Induced in G2/M Phase Largely Follows the Rules of Origin Selection, But Not the Rules of Origin Activation, That Govern S Phase Replication

We have taken advantage of our microarray CGH assay to perform a genome-wide analysis of eukaryotic re-replication. This comprehensive analysis has allowed us to examine several key tenets of the current model for replication control. One important tenet is that reinitiation that arises from deregulation of ORC, Mcm2-7, and Cdc6 occur from sites of pre-RC formation in S phase. The overall concordance of mapped re-replication origins with pro-ARSS suggests that the reinitiation occurs at sites that normally assemble pre-RCs for S phase replication. Although current limitations of the resolution of microarray data prevent a precise match of replication and re-replication origins, in the few cases where this has been directly tested by 2-D gel electrophoresis or deletion analysis (Figure 7 and Nguyen *et al.*, 2001), we have confirmed that this is, in fact, the case. Thus, the sequence determinants that select potential origins in S phase appear to be conserved during re-replication.

In contrast to the selection of potential origins, the activation of these origins during re-replication in G2/M phase differs considerably from origin activation during replication in S phase. During S phase replication, poorly understood chromosomal determinant specify which potential origins are activated early, which are activated late, and which remain latent. During re-replication in G2/M phase, all three classes are among the 106 origins that reinitiate, and there is no correlation between the time/efficiency pro-ARSS replicate in S phase and the efficiency with which they re-replicate in G2/M phase. These results suggest that the chromosomal determinants governing S phase origin activation are not preserved during G2/M phase re-replication. Such a conclusion is consistent with the finding that the temporal program for origin firing in S phase is lost by G2/M phase and must be reestablished upon entry into each new cell cycle (Raghuraman *et al.*, 1997).

The Block to Re-replication Uses a Common Fundamental Strategy Implemented in a Dynamic Manner Across the Cell Cycle

Another important tenet of the replication control model is that the blocks to re-replication in S, G2, and M phase use the

phase re-replication profile shown for chromosome III (gray line). The *MC_{2A}* G2/M phase re-replication profile from Figure 5E is replotted for comparison (black line). The locations of pro-ARSS mapped by Wyrick *et al.* (2001) (gray triangles), and the centromere (black circle) are plotted along the X-axis. (C) *Cdc7* kinase is required for re-replication induced in *MC_{2A}* cells. The *MC_{2A}* strain YJL4489, the congenic *MC_{2A}-cdc7* strain YJL5821 (*MCM7-2NLS pGAL1-Δntcd6-2A pMET3-HA3-CDC20 cdc7-1*), and their respective controls, the M strain YJL4486 and the M-*cdc7* strain YJL5816 (*MCM7-2NLS pGAL1 pMET3-HA3-CDC20 cdc7-1*) were induced with galactose as described in Figure 6A, except the initial arrest was performed at 23°C, and the arrested cells were shifted to 35°C for 1 h, before the addition of galactose. Genomic DNA was isolated 4 h after galactose addition and competitively hybridized (*MC_{2A}* vs. M and *MC_{2A}-cdc7* vs. M-*cdc7*) as described in Figure 1A. Re-replication profiles for the *MC_{2A}* (black line) and *MC_{2A}-cdc7* (gray line) strains are shown for chromosome III. Locations of pro-ARSS mapped by Wyrick *et al.* (2001) (gray triangles), and the centromere (black circle) are plotted along the X-axis.

same fundamental strategy of preventing pre-RC reassembly. Deregulating the mechanisms that prevent this reassembly in any of these cell cycle phases should thus lead to re-replication. Studies in human, *Drosophila*, and *Caenorhabditis elegans* that deregulate geminin (Melixetian *et al.*, 2004), Cdt1 (Thomer *et al.*, 2004), and Cul-4 (which stabilizes Cdt1; Zhong *et al.*, 2003), respectively, have inferred that re-replication can occur within S phase based on evidence of a prolonged S phase. In this study, we directly demonstrate that cells can reinitiate replication at multiple origins while the first round of replication is still ongoing. Thus, we establish that mechanisms used to prevent re-replication in G2/M phase also prevent re-replication within S phase.

Despite sharing common mechanisms to carry out the same fundamental strategy, the block to re-replication in S phase and G2/M phase are not identical. Two differences are readily apparent when comparing cells re-replicating through S phase during a G1 release with cells re-replicating at a G2/M phase arrest. The first difference is the bias toward reinitiation of early origins that is only observed in the G1 release experiment. The simplest explanation for this bias is suggested by the S phase re-replication profiles, which show reinitiation at early origins occurring before late origins have had a chance to fire. These observations suggest that early origins clear their replication pre-RCs sooner and are more available for pre-RC reassembly during S phase, although other explanations for this bias cannot be ruled out.

The second difference between the G1 release and G2/M phase re-replication is that the amount of re-replication induced during the G1 release was greater than the amount induced in G2/M phase in both the OMC and OC strains. This difference can be observed by flow cytometry but is most striking when G1 release and G2/M phase re-replication profiles are compared. There are a growing number of examples of mechanisms that vary in their efficacy across the cell cycle, such as Cdc6 degradation in budding yeast (Perkins *et al.*, 2001), Cdt1 degradation in *Xenopus* and humans (Nishitani *et al.*, 2004; Arias and Walter, 2005; Li and Blow, 2005; Yoshida *et al.*, 2005), and geminin inhibition in human cells (Ballabeni *et al.*, 2004). Together these results indicate that the block to re-replication is dynamic with the number and relative contribution of regulatory mechanisms implementing the block changing during the cell cycle.

What Is Limiting Re-replication?

A key difference between re-replication and replication in the OMC strain is that a significantly smaller number of origins initiate efficiently during re-replication (106 vs. 193). This reduction in origin firing likely contributes to the limited re-replication observed in the OMC strain and suggests that additional mechanisms are still restraining reinitiation. Consistent with both notions, additional mechanisms inhibiting ORC (by CDK binding to Orc6; Wilmes *et al.*, 2004) and Cdc6 (by CDK binding to the N-terminus of phosphorylated Cdc6; Mimura *et al.*, 2004) have recently been identified in budding yeast. The latter mechanism is already disrupted in the OMC strain because of the N-terminal deletion of Cdc6. Disrupting the former mechanism in the OMC background moderately enhances re-replication, but this re-replication is still restrained (Wilmes *et al.*, 2004; Tanny *et al.*, 2006), suggesting that still more re-replication controls remain to be identified.

The reduced number of reinitiating pro-ARSs, however, may not be the only factor limiting re-replication. Previous work suggests that a single replication fork should be able to replicate 100–200 kb (Dershawitz and Newlon, 1993; van Brabant *et al.*, 2001). Our re-replicating profiles show that the

amount of DNA synthesis associated with many reinitiating origins is significantly reduced 100–200 kb away from these origins (Supplementary Figure S7). These data suggest that re-replicating forks may not be able to progress as far as replicating forks, although a more direct analysis of fork movement will be needed to confirm this hypothesis.

Multiple Nonredundant Mechanisms Work in Combination to Reduce the Probability of Re-replication

We previously showed that we could reliably detect G2/M phase re-replication by flow cytometry in the OMC strain when ORC, Mcm2-7, and Cdc6 are deregulated, but not when only two of the three proteins were deregulated (Nguyen *et al.*, 2001). Since then, there have been many other examples where multiple replication controls had to be disrupted to detect re-replication (reviewed in Diffley, 2004; Blow and Dutta, 2005). These observations have led to the presumption that the eukaryotic replication controls are redundant. We favor an alternative view that replication controls are not redundant and that disruption of one or a few of controls can lead to low levels of re-replication.

Failure to detect this re-replication has been due to the insensitivity of standard replication assays. In support of the view, the more sensitive microarray CGH assay used in this study was able to detect G2/M phase re-replication in the OC and MC_{2A} strains. We did not detect re-replication when only a single mechanism was disrupted, but we note that the microarray CGH assay has its own detection limits and may have difficulty detecting rare or sporadic replication events. The development of even more sensitive single-cell assays that can detect these rare re-replication events may reveal that the chance of re-replication occurring is increased when ORC, Mcm2-7, or Cdc6 is individually deregulated.

Our findings support a model in which the block to re-replication is provided by a patchwork of many mechanisms, each of which contributes to a portion of the block by reducing the probability that re-replication will occur within a cell cycle. The combined action of all these mechanisms is needed to reduce the probability to such low levels that re-replication events become exceedingly rare and virtually prohibited. Successive disruption of these mechanisms does not lead to a sudden collapse of the block after a threshold of deregulation is reached, but instead results in a gradual erosion of the block manifested by incrementally higher frequencies and/or levels of limited re-replication. Because all mechanisms contribute in some way to the block, more than one mechanism or combination of mechanisms can be overridden to generate detectable re-replication. Hence, the fact that disruption of a mechanism is sufficient to induce limited re-replication does not make it the critical or dominant mechanism in the block to re-replication.

Levels of Re-replication Likely to Contribute to Genomic Instability and Tumorigenesis May Not Be Detectable by Most Currently Available Assays

Because genomic instability is associated with, and possibly facilitates, tumorigenesis, there has been much interest in understanding the derangements in DNA metabolism and cell cycle control that can cause genomic instability. Re-replication is a potential source of genomic instability both because it produces extra copies of chromosomal segments and because it generates DNA damage and/or replication stress (Melixetian *et al.*, 2004; Zhu *et al.*, 2004; Archambault *et al.*, 2005; Green and Li, 2005). Re-replication has also been potentially linked to tumorigenesis by the observation that overexpression of Cdt1, which can contribute to re-replication (reviewed in Blow and Dutta, 2005), can transform

NIH3T3 cells into tumorigenic cells (Arentson *et al.*, 2002). However, two considerations have raised concerns about the biological relevance of these potential connections. First, if replication controls are highly redundant, the probability that a cell will spontaneously acquire the multiple disruptions needed to induce re-replication will be extremely small. Second, we and others have shown that cells undergoing overt re-replication experience extensive inviability (Jallepalli *et al.*, 1997; Yanow *et al.*, 2001; Wilmes *et al.*, 2004; Green and Li, 2005) or apoptosis (Vaziri *et al.*, 2003; Thomer *et al.*, 2004), making cell death a more likely outcome than genomic instability or tumorigenesis.

Our results in this study counter the first concern by challenging the concept of redundancy in replication control and showing that very low levels of re-replication can still be observed when fewer controls are disrupted. We also have evidence that lower levels of re-replication induce lower levels of inviability (unpublished data), diminishing the second concern. Consequently, we suggest that re-replication at levels well below current detection limits may occur with greater frequency than previously anticipated and that genomic instability may arise from these low, nonlethal levels of re-replication.

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Loss of DNA replication control is a potent inducer of gene amplification

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Running Title: DNA re-replication generates gene amplifications

[Key words: DNA replication, gene amplification, yeast, re-replication, rereplication, copy number variation, breakage fusion bridge cycles]

Eukaryotic cells use a multitude of overlapping mechanisms to ensure that their DNA is virtually never re-replicated within a single cell cycle¹. This extensive regulation is thought to protect cells from heritable genomic changes that could, in principle, arise from re-replication. Direct evidence for such genomic alterations has been lacking, however. Here we show in the budding yeast *Saccharomyces cerevisiae* that re-replication arising from loss of DNA replication control induces a high frequency of gene amplification, a genomic alteration often linked to cancers.

The amplified units, or amplicons, consist of large internal chromosomal segments up to several hundred kilobases long that are bounded by repetitive sequences and intrachromosomally arrayed in direct head-to-tail orientation. The high incidence of these segmental amplifications appears to be specific to re-replication, as they are not observed with appreciable frequency when S phase DNA replication is impaired or DNA is directly damaged. While similarly arrayed amplicons in direct repeat have been observed in tumors², these structures have eluded explanation by the prevailing breakage-fusion-bridge (BFB) model for gene amplification, which predicts formation of indirect repeats adjacent to telomeric deletions³. We thus propose that loss of replication control should be considered a potential source of the genomic instability associated with carcinogenesis.

Many proteins involved in the initiation of eukaryotic DNA replication are tightly regulated to minimize the probability that re-initiation within a single cell cycle occurs at any of the hundreds to thousands of replication origins in a genome¹. Although such exquisitely tight control is presumed to be necessary because re-replication could

conceivably poses a threat to genome stability, this premise has never been experimentally tested. Hence, re-replication has not been seriously considered as a source of genomic mutability, either on an evolutionary time scale or the more accelerated time scale associated with carcinogenesis.

Our recently acquired ability to disrupt replication control in budding yeast^{4,5} has allowed us to examine whether re-replication can promote heritable genomic alterations. Because re-replication synthesizes extra gene copies, we have first asked whether re-replication can induce gene amplification, a heritable increase in the copy number of genes. Notably, gene amplification of known or suspected oncogenes is associated with many tumors².

The mechanism behind many gene amplification events is poorly understood². Gene amplifications can be intrachromosomal or extrachromosomal and the amplified structures can be quite diverse. The most molecularly defined model for intrachromosomal gene amplification, the breakage-fusion-bridge (BFB) model (see Supplemental Figure 1) leads to the following three structural features: (1) amplicons in inverted orientation, (2) deletion of DNA telomeric to the amplified region, and (3) mitotic bridges arising from bipolar tension on dicentric chromosomes³. Such structural hallmarks have been documented most carefully in cell culture systems where gene amplification is induced by drug selection⁶⁻⁸, but they have also been observed in some tumors^{2,9}.

An increasing number of tumors, however, have been shown to contain amplified structures incompatible with the BFB model¹⁰. For example, some of the classic examples of amplified oncogenes in tumors, such as MYCN in neuroblastomas¹¹ and

ERBB2 in breast, ovarian and gastric cancers¹² are arrayed in direct repeats. We show here that re-replication induced gene amplification can account for intrachromosomal amplicons arranged in direct repeat and provides a new gene amplification paradigm that can complement the BFB model.

To detect gene amplification arising from DNA re-replication in the budding yeast *Saccharomyces cerevisiae*, we adapted a colony color assay that monitors the copy number of an *ade3-2p* reporter gene. In this assay, cells with a single copy of *ade3-2p* allele turn pink, and cells with two or more copies turn red¹³. To examine the effect of re-replicating this reporter gene, we constructed an *ade3-2p* reporter cassette (Fig 1A) containing *ade3-2p* and *ARS317*, the primary origin that re-initiates replication in strains deregulated for Mcm2-7 and Cdc6 (*MC2A* strain background)⁵. We integrated the cassette at either 567 kb or 1089 kb from the left end of chromosome IV and deleted *ARS317* from its endogenous locus. Re-initiation of *ARS317* is conditional in the resulting strains (induced by galactose and repressed by dextrose), allowing us to examine the consequences of a transient pulse of re-replication at a defined genomic locus.

In Fig 1B we confirm that re-replication of the *ade3-2p* cassette is efficient and dependent on *ARS317*. Cells arrested in G2/M with nocodazole were induced to re-replicate by addition of galactose for three hours then harvested to examine their DNA re-replication by array comparative genomic hybridization (array CGH) against DNA from a non-replicating reference strain. In the resulting re-replication profiles, peak position indicates the site of re-initiation, peak height represents the efficiency of re-initiation, and peak width reflects the size distribution of the re-replication bubbles⁵. Re-initiation at

the *ade3-2p* reporter cassettes increased their DNA content by 50% (2C to ~3C) (Fig 1B), which is similar to the efficiency observed for *ARS317* at its endogenous locus⁵.

Following three hours of induced re-replication, we plated single cells onto medium that repressed further re-initiation and allowed proper color development of the resulting colonies. We reasoned that, in some cells or their immediate descendants, the re-replication bubble containing a duplication of *ade3-2p* would be converted to a stable heritable structure, causing all subsequent progeny to turn red. Fig 1C shows an example of a colony with a quarter red sector that presumably arose when one cell acquired a stable duplication of *ade3-2p* at the four-cell stage. In order to focus on stable duplications that occurred soon after re-replication was induced, we scored pink colonies with half, quarter and eighth red sectors. We then colony purified cells from the red sectors of these colonies to analyze their genomic structure.

Galactose induction of re-replication in *MC2A* cells with the *ade3-2p* cassette integrated 567 kb from the left end of chromosome IV (YJL6558) caused a striking increase in red sectors to 3.6% of all colonies (Fig 2A). No increase was observed in a congenic strain that could not re-replicate because it lacked the inducible Cdc6 (YJL6974). Moreover, re-replicating cells containing an integrated *ade3-2p* cassette lacking *ARS317* (YJL6555) showed only a small increase in sectoring, suggesting re-initiation from the cassette is necessary for efficient sector formation. The few red sectors induced in cells with a cassette lacking *ARS317* were likely due to sporadic and inefficient re-replication that occurs throughout the genome in the *MC2A* strain⁵. Re-replication also induced significant colony sectoring when the *ade3-2p* cassette was located 1089kb from the left end of chromosome IV (Supplemental Fig 2A).

We selected 24 red sectors induced by re-replication from the *ade3-2p* cassette at 567kb to determine their extent and amount of amplification using array CGH. Genomic DNA from these red sector cells was hybridized against genomic DNA from a reference strain with a non-amplified genome (Fig 2B). Of the 24 red sectors, 20 had at least two copies of the chromosomal segment spanning the *ade3-2p* cassette, confirming that gene amplification had occurred in most of the sectored colonies. Since none of our amplified chromosomal segments contained telomeres or centromeres, we describe them as segmental amplifications. Such amplifications must be integrated in an existing chromosome to be stably maintained and detected in our colony-sectoring screen.

The boundaries of the amplified regions appeared to coincide with Ty elements or LTR sequences (long terminal repeat segments that remain after Ty excision) that have been mapped in the *Saccharomyces* Genome Database¹⁴. These dispersed repetitive elements have been found at the junctions of many deletions and translocations observed in *S. cerevisiae*¹⁵, and such junctions are thought to arise from homologous recombination between these elements. Eighteen of the 20 amplified regions were bounded by the nearest Ty elements to either side of the *ade3-2p* cassette. The remaining amplified regions were bounded at one or both ends by a more distal LTR or Ty element.

When the *ade3-2p* cassette was integrated at 1089kb from the left end of chromosome IV (YJL6561), 16 of the 24 red sectors induced by re-replication had segmental amplifications spanning the cassette (Supplemental Fig 2B). Although the boundaries of these amplifications were more varied than those derived from the *ade3-2p* cassette at 567 kb (YJL6558), they retained three shared features. First, the boundaries enclosed amplified segments on the order of 100-400 kb long, roughly the width of the

re-replication peaks. Second, all but one boundary mapped near a Ty or LTR element. Third, there was a preference for elements that were close to the *ade3-2p* cassette, where re-replication initiates. These properties are consistent with a scenario in which segmental amplifications arise from homologous recombination of re-replicated Ty or LTR elements (see below).

To determine which chromosomes contained the segmental amplifications, we separated chromosomes using pulsed field gel electrophoresis (PFGE) and probed for the presence of the *ADE3* gene (Fig 2C). This probe hybridizes to both the endogenous mutant *ade3* allele on chromosome VII as well as the *ade3-2p* cassette integrated in chromosome IV (Fig 2C, lanes 1 and 26, parental strains). The additional amplified segments are large enough to noticeably decrease the mobility of the host chromosome. All of the 20 strains with segmental amplifications derived from the *ade3-2p* cassette at 567 kb showed an increase in chromosome IV size consistent with the size and copy number of their amplicons. (Fig 2C, lanes 2-25). Importantly, no other chromosome besides IV (site of *ade3-2p* cassette) and VII (site of endogenous *ade3*) were detected by the *ADE3* probe, indicating that all amplified segments were only integrated in chromosome IV.

To determine the precise position of these amplified segments in chromosome IV, we tested the hypothesis that these segments are arranged in tandem at the original locus. Tandem duplications can be arranged in three possible arrangements: head to tail, head to head and tail to tail, each generating a unique set of junctions and boundaries that can be distinguished by PCR (Fig. 2D). Of the 20 red sectors containing an amplification of the *ade3-2p* cassette at 567 kb, 19 generated PCR products consistent with a tandem head-to-

tail duplication at the original locus. Moreover, the sizes of the PCR products were consistent with the expected presence of Ty or LTR elements at the amplicon boundaries, an expectation confirmed by sequencing (data not shown). Similar PCR analysis of the amplification structures induced by re-replication from the 1089 kb locus also indicated a head-to-tail orientation of duplicate amplicons (data not shown). Thus, re-replication can induce segmental amplification with the amplicons in direct repeat and bounded by Ty or LTR elements.

Defects in S phase replication due to partial depletion of replication proteins or insertion of palindromic DNA structures have been implicated in gene duplication or amplification events^{16,17}. The frequency of these amplification events (5×10^{-4} and 3×10^{-5} , respectively) is at, or lower than, the background of our sectoring assay, and the resulting amplification structures (extrachromosomal elements, chromosome arm duplications and inverted repeats) differ from those we characterized. Nonetheless, these observations raise the question of whether re-replication induced gene amplification is simply a secondary consequence of disrupted replication or DNA damage, particularly because the re-replication surrounding *ARS317* in the *MC2A* background is limited (Fig 1B) and because re-replication leads to DNA damage^{1,18}. To address this question, we asked whether either S phase disruption or DNA damage could induce detectable gene amplification in our sectoring screen

To disrupt S phase replication we used mutants that affect various steps of replication. Diploid strains homozygous for the temperature sensitive alleles *cdc6-1*, *cdc7-1*, *cdc9-1*, or *cdc17-1* were shifted to either nonpermissive (36° C for 3 hours) or semi-permissive (30° C for 6 hours) conditions. We also treated cycling cells with

hydroxyurea, a drug that depletes nucleotides, for 3 hr (0.2M) or 6 hr (0.1M). Little, if any, increase in red sector formation was observed in the sectoring assay after these treatments (Fig 3A, B). To induce DNA damage we treated diploid cells for 3 hr with two different concentrations of the DNA damaging agent phleomycin before assaying for red sectors (Fig 3C). Although DNA damage did cause a small increase in the frequency of red sectors, most of the sectors appeared qualitatively less red than those due to re-replication (data not shown), and microarray CGH on 24 of the sectors showed that none displayed any segmental duplication of chromosome IV (Fig 3D). Thus the estimated frequency of actual head to tail gene amplification events shows little induction by DNA damage (Fig 3E). Together, these data indicate that the high frequency of tandem direct gene amplification events is specific to re-replication.

What is it about re-replication that gives it a special propensity to generate segmental amplifications in direct tandem repeat? One possibility is that re-replicating forks are particularly susceptible to breakage and re-replication bubbles provide an optimal context for break repair to create direct repeat amplifications. Figure 4 shows a working model that incorporates these ideas. In this model, re-replication past repetitive elements at opposite ends of a bubble followed by breaks in trans at the two forks could lead to homologous recombination between the two opposing elements. Such a recombination event would convert the two parallel re-replicated arms into serial direct repeats bounded by repetitive elements. Future experiments to test this and related models will need to detect and structurally characterize the broken chromosomal intermediates predicted by the models.

Our observation that re-replication is a potent inducer of gene amplification suggest that loss of replication control may be a more prominent contributor to carcinogenesis than previously appreciated. Interestingly, elevated expression of replication proteins has been observed in a number of cancers¹⁹. Although this elevation could simply be secondary to the increased proliferation of tumorigenic cells, our results raise the possibility that in some cases this elevation might have a causative role. Encouraging such speculation is the observation that overexpression of the replication protein, Cdt1, which can induce re-replication in humans, *Drosophila melanogaster*, *Xenopus laevis*, *Caenorhabditis elegans*¹, somehow promotes carcinogenesis in mice^{20,21}. Although re-replication was not readily apparent in these mouse cells, we note that only barely detectable levels of re-replication may be capable of promoting carcinogenesis, as overt re-replication leads to massive cell death^{18,22-25}. Finally, our experimental design and sectoring assay was best suited for detecting intrachromosomal amplifications. Alterations of the design and assay may reveal other types of genomic instability induced by re-replication such as extrachromosomal amplifications, segmental deletions, and missegregation (from re-replication of centromeres).

Increases in gene copy number are also important for evolution, as gene duplication allows for functional divergence of the duplicates²⁶. An estimated 30 to 60% of eukaryotic genes arose from gene duplication events²⁷, and some of these events may have been similar to the segmental duplications we observed in this report. In addition, the recent observation that as much as 12% of the human genome displays copy number variation (CNV) within a set of <300 individuals²⁸, has raised the possibility that copy number increases (and decreases) may be an important source of phenotypic variation,

the substrate for evolutionary selection. The mechanism by which many of these CNVs are generated is unknown, since precise structures for most have not been determined. Nonetheless, we suggest that sporadic re-replication should be considered as a possible driving force in evolution and phenotypic variation as well as carcinogenesis

Methods

Strains (Supplemental Table 1) and plasmids (Supplemental Table 2) were constructed as described in Supplemental Methods. Oligonucleotide sequences are described in Supplemental Table 3. Details of strains derived from red sectors are described in Supplemental Table 4. Cells were grown in or on YEP or synthetic complete (SC) medium²⁹ supplemented with 2% dextrose (wt/vol) or 3% raffinose (wt/vol) + 0.05% dextrose (wt/vol). All experiments were performed at 30°C except where noted.

To obtain reproducible induction of re-replication, cells were inoculated from a fresh unsaturated culture containing 2% dextrose into a culture containing 3% raffinose + 0.05% dextrose and grown for 12–15 h the night before the experiment. After cells were arrested in G2/M with 15ug/ml nocodazole, the *GAL1* promoter (*pGAL1*) was induced by addition of 2% galactose for 3 hours. To perturb S phase replication, the indicated mutant strains were grown overnight in YEPD at 23°C, then shifted to 36°C or 30°C for 3 or 6 hours, respectively or wild type strain was grown in YEPD at 30°C overnight then 0.2M or 0.1M hydroxyurea was added for 3 or 6 hours, respectively. To induce DNA damage, cells were grown in YEPD at 30°C overnight then 0.2ug/ml or 2ug/ml phleomycin was added for 3 hours.

To score the frequency of red sectors, ~200 colonies were plated onto SDC plates containing 0.5x adenine. Temperature sensitive strains were grown for 7 days at 23°C and other strains were grown at 30°C for 5 days, then 23°C for 3 days to optimize colony color development. Plates were randomized and scored blind. Red sectors were counted if: 1) the sectors were greater than 1/8 of the colony, 2) darker red than the neighboring colonies (ie, not a pink sector in a nearly white colony) and 3) the junctions between the red sector and pink colony were largely straight, to minimize sectors due to poor growth. The frequency of sectored colonies was determined by dividing the total sector counts by the total number of viable colonies.

To assess the extent and amount of amplification, comparative genomic hybridization (array CGH) was performed as described⁵ without application of the smoothing algorithm.

Cells were prepared for pulsed field gel electrophoresis as described¹⁸. Plugs were cut in half and loaded on a 1% SeaKem LE agarose (wt/vol) gel in 1x TAE (40 mM Tris, 40 mM acetate, and 2 mM EDTA, pH 8.0). The gel was electrophoresed in 14°C 1x TAE on a CHEF DR-III system with a switch time of 500 s, run time of 48 h, voltage of 3 V, and angle of 106°. The DNA was transferred as described¹⁸ and probed with an *ADE3* probe generated with OJL1757 and OJL1758 (Supplemental Table 3).

Use of PCR to analyze novel genomic DNA junctions is described in the Supplemental Methods.

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Figure 1 – Gene amplification assay

- A) An *ade3-2p* reporter cassette consisting of the copy number reporter *ade3-2p*, the re-initiating origin *ARS317*, and the selectable marker kanMX was inserted into the genomic loci of interest via homologous recombination. Cassettes lacking the origin were inserted in control strains.
- B) *ARS317* in the *ade3-2p* cassette is sufficient for re-initiation at ectopic loci. The reporter cassette was integrated at two genomic loci in a strain deleted for the endogenous *ARS317*. Cells were arrested in G2/M phase, galactose was added for 3 hours to induce Δnt-cdc6-2A, and genomic DNA was then isolated for replication analysis by array competitive genomic hybridization. Top panel: YJL6557 contains an *ade3-2p* cassette lacking *ARS317* integrated 1089kb from the left end of chromosome IV. Middle panel: YJL6558 contains an *ade3-2p* cassette inserted 567kb from the left end of chromosome IV. Bottom panel: YJL6561 contains an *ade3-2p* cassette inserted 1089kb from the left end of chromosome IV.
- C) Schematic of gene amplification screen. Cells were induced to re-replicate for three hours at a G2/M arrest then plated for single colonies on plates that allow colony color development. Shown is an example of a colony where stable heritable amplification of the *ade3-2p* reporter was acquired by one cell at the four-cell stage, resulting in a pink colony with a red quarter sector. Colonies with 1/2, 1/4, and 1/8 red sectors were streaked to colony purify red cells. Those sectors that were confirmed by the streaks were quantified and their DNA analyzed further for genomic copy number variation.

Figure 2 – Re-replication induces segmental duplications in head-to-tail orientation

A) Re-replication induces colony sectoring. YJL6974, YJL6555, and YJL6558 were assayed for gene amplification frequencies as described in Figure 1C.

All strains were *MCM7-2NLS ars317Δ ade3 ade2 Chr IV 567kb::ade3-2p cassette*.

Distinguishing alleles are indicated, with *ARS317* referring to the presence of the origin in the *ade3-2p* cassette. The mean and standard error of the mean of at least two independent experiments is shown.

B) Sectors contain segmental amplifications. 24 sectors identified in Figure 2A from YJL6558 were analyzed by array CGH using non-replicating reference DNA from YJL6032. Four types of copy number variations on chromosome IV (1-4) were observed. For each type, a representative copy number profile and the number of sectors observed is shown. Four sectors showed no evidence of increased *ade3-2p* copy number.

C) Re-replication induces intrachromosomal gene amplifications. Chromosomes from the 24 sectors analyzed in Figure 2B were separated by PFGE and probed for *ADE3*, which detects both the endogenous *ade3* locus on chromosome VII and the *ade3-2p* reporter gene integrated on chromosome IV. Lane 1 and 26: YJL6558 parental strain before induction of re-replication. Lanes 2-25: Sectored isolates 1-24 respectively. Doublet pattern for chromosome IV in lanes 5 and 6 is consistent with a partial loss of the segmental amplification from the population.

D) Amplicons are tandemly arrayed in direct head-to-tail orientation. Schematic shows structure of unamplified amplicon unit and the three possible orientations for tandemly duplicated amplicons. Predicted PCR junction fragments spanning the boundaries of each amplicon structure are shown for the various combinations of primers

displayed (+ PCR product expected; - no PCR product expected). The parental strain YJL6558 and 20 derivative strains that were shown in Figure 2C to contain segmental duplication of the *ade3-2p* reporter were subject to this PCR analysis. 19 of 20 (all of type 1, type 2, and type 4 from Figure 2B) generated a pattern of PCR products only compatible with tandem amplicons in head-to-tail orientation.

Figure 3 – Underreplication and DNA damage does not lead to significant gene amplification

- A) Diploid strains YJL7002 (WT), YJL7003 (*cdc6-1/cdc6-1*), YJL7085 (*cdc7-1/cdc7-1*), YJL7005 (*cdc9-1/cdc9-1*), and YJL7006 (*cdc17-1/cdc17-1*) were grown exponentially at 23° C then shifted to 36° C for 3 hours or 30° C for 6 hours to perturb replication. The effectiveness of this perturbation was monitored by the percent of cells containing large buds. The frequency of red sectors was determined in at least two independent experiments and the mean and standard error of the mean are shown. The frequency due to re-replication of YJL6558 (described in Figure 2A) is provided for reference.
- B) Diploid YJL7007 (WT) grown exponentially at 30 C was treated with hydroxyurea for the times and concentrations shown and analyzed as described in A.
- C) DNA damage induces a small increase in sectoring frequency. Exponentially growing diploid YJL7007 (WT) cells were treated with the indicated concentration of phleomycin for 3 hours and the frequency of red sectors was obtained as described in Figure 1C. The mean and standard error of the mean of at least two independent

experiments are shown. The frequency due to re-replication of YJL6558 (described in Figure 2A) is shown for reference.

D) Sectors induced by DNA damage do not contain any segmental amplification spanning the *ade3-2p* reporter gene. 24 red sectors obtained from treatment of YJL7007 with 20 µg/ml phleomycin (described in Figure 3C) were analyzed by array CGH. All displayed the representative copy number profile of chromosome IV shown in the figure.

E) DNA damage does not significantly induce the head to tail gene amplification events that are observed following re-replication. The red sector frequencies induced by re-replication of YJL6558 (from Figure 2A) were multiplied by 19/24, the fraction of sectors that were confirmed to contain a head-to-tail gene amplification in Figure 2D. The red sector frequency induced by phleomycin treatment of YJL7007 were multiplied by 1/24, the minimum fraction of these sectors that could have been observed to have a head-to-tail gene amplification.

Figure 4 – Working model for re-replication induced gene amplification

See text for description of model. Open arrowheads represent repetitive sequences while grey arrows are regions that will be amplified.

Supplemental Figure 1 – Breakage fusion bridge cycles

A schematic of breakage fusion bridge cycles is shown. Breakage through both sister chromatids, or a break in G1 phase of the cell cycle, (upper left) can result in fusion of the two sisters (upper right). This results in a dicentric chromosome, a mitotic bridge resulting from attempted segregation (lower right), then resolution by breakage. The

resulting asymmetric chromosomes result in duplication of the region centromeric to the break and loss of the region telomeric. The amplicon is arranged in an inverted orientation (lower left). Replication of this DNA results in two sister chromatids each with a break – allowing the cycle to be repeated (upper left) until a telomere is captured.

Supplemental Figure 2 – Re-replication induces segmental duplications at another locus

A) Re-replication induces gene amplifications. YJL6977, YJL6557, and YJL6561 were assayed for gene amplification frequencies as described in Figure 1C.

All strains were *MCM7-2NLS ars317Δ ade3 ade2 Chr IV 1089kb::ade3-2p cassette*.

Distinguishing alleles are indicated, with *ARS317* referring to the presence of the origin in the *ade3-2p* cassette. The mean and standard error of the mean of at least two independent experiments is shown.

B) Amplification events induced by re-replication involve segmental duplications.

Representative sectors identified in Supplemental Figure 2A from YJL6561 were analyzed by array CGH using non-replicating reference DNA from YJL6032.

C) Summary of segmental duplication amplification events induced by re-replication. 24 sectors identified in Supplemental Figure 2A were analyzed by array CGH and schematics of the amplicons are shown. Bold lines indicate the amplified region for each class of amplicon. Asterisks indicate breakpoints of amplicons that appeared to occur at long terminal repeats, rather than full Ty elements. The plus symbol indicates the one breakpoint that does not appear to correspond to a previously described Ty or long terminal repeat.

Figure 1

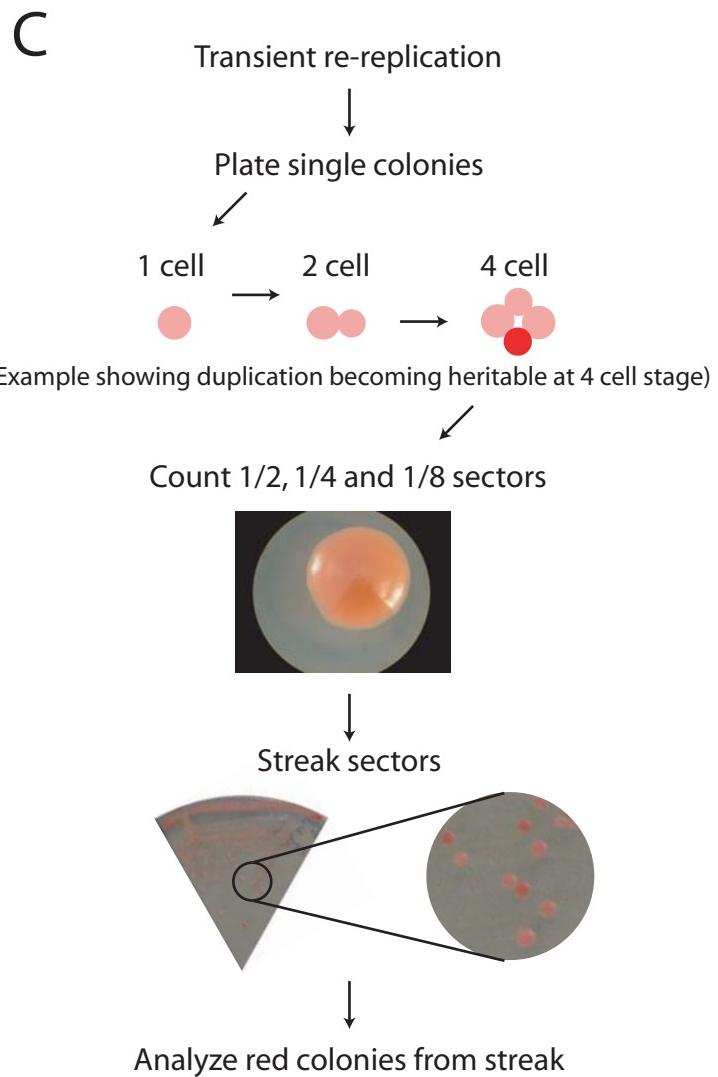
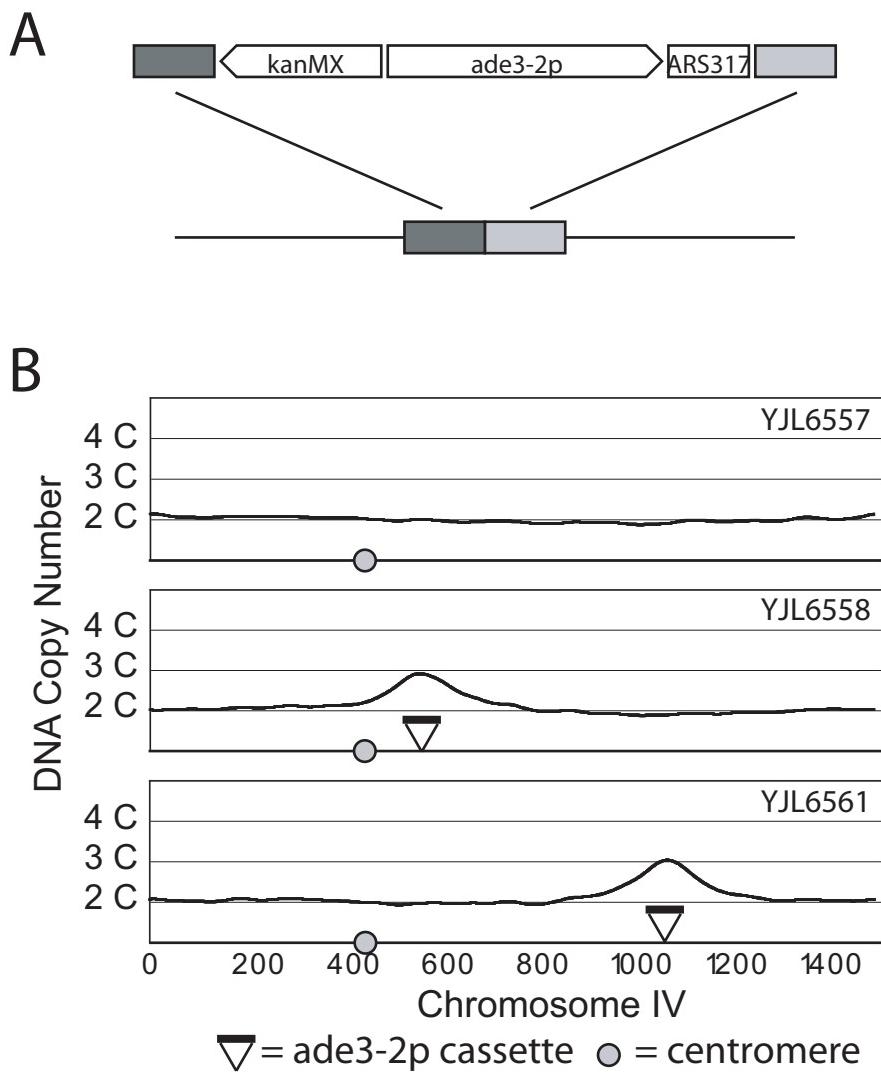


Figure 2

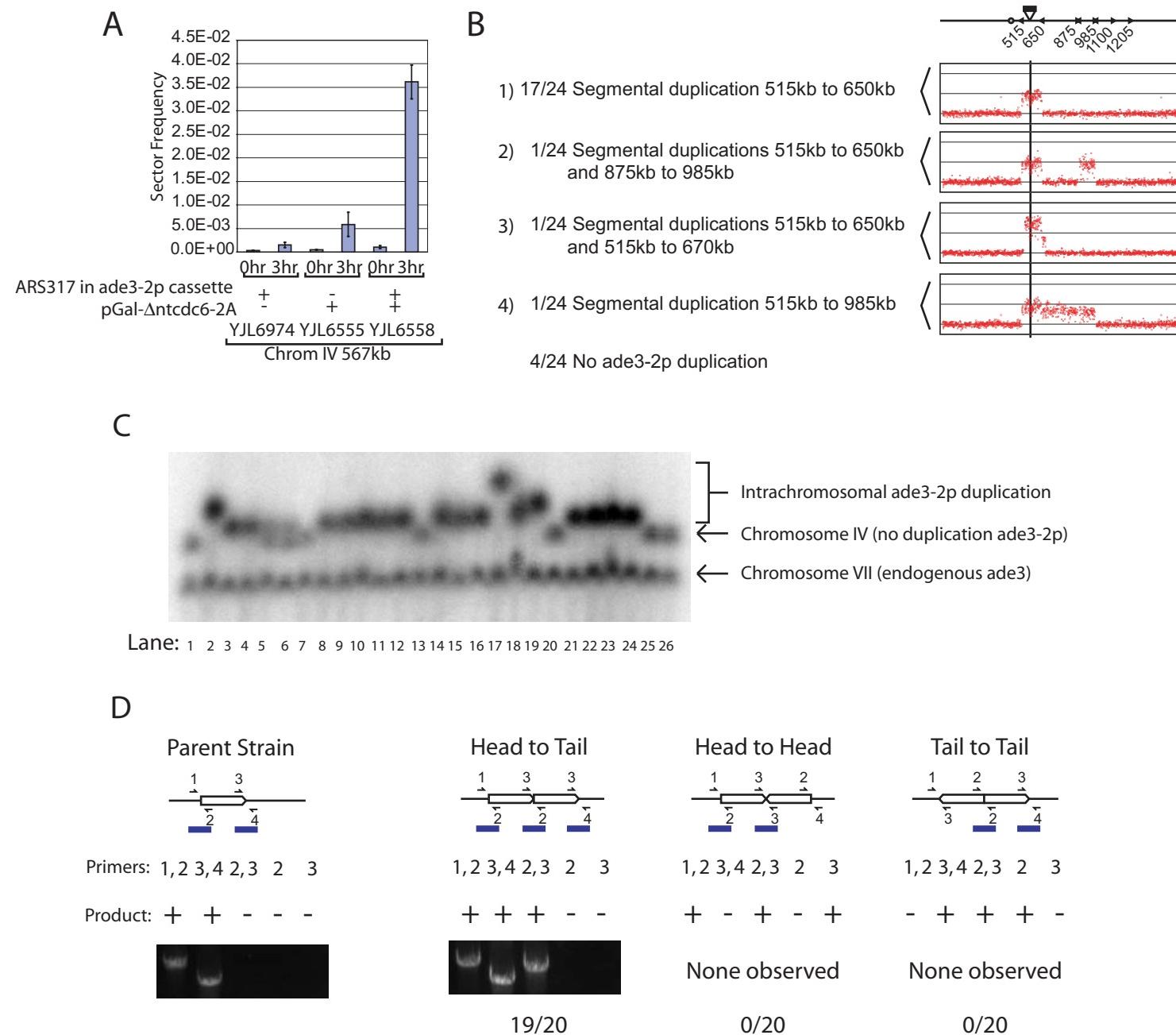


Figure 3

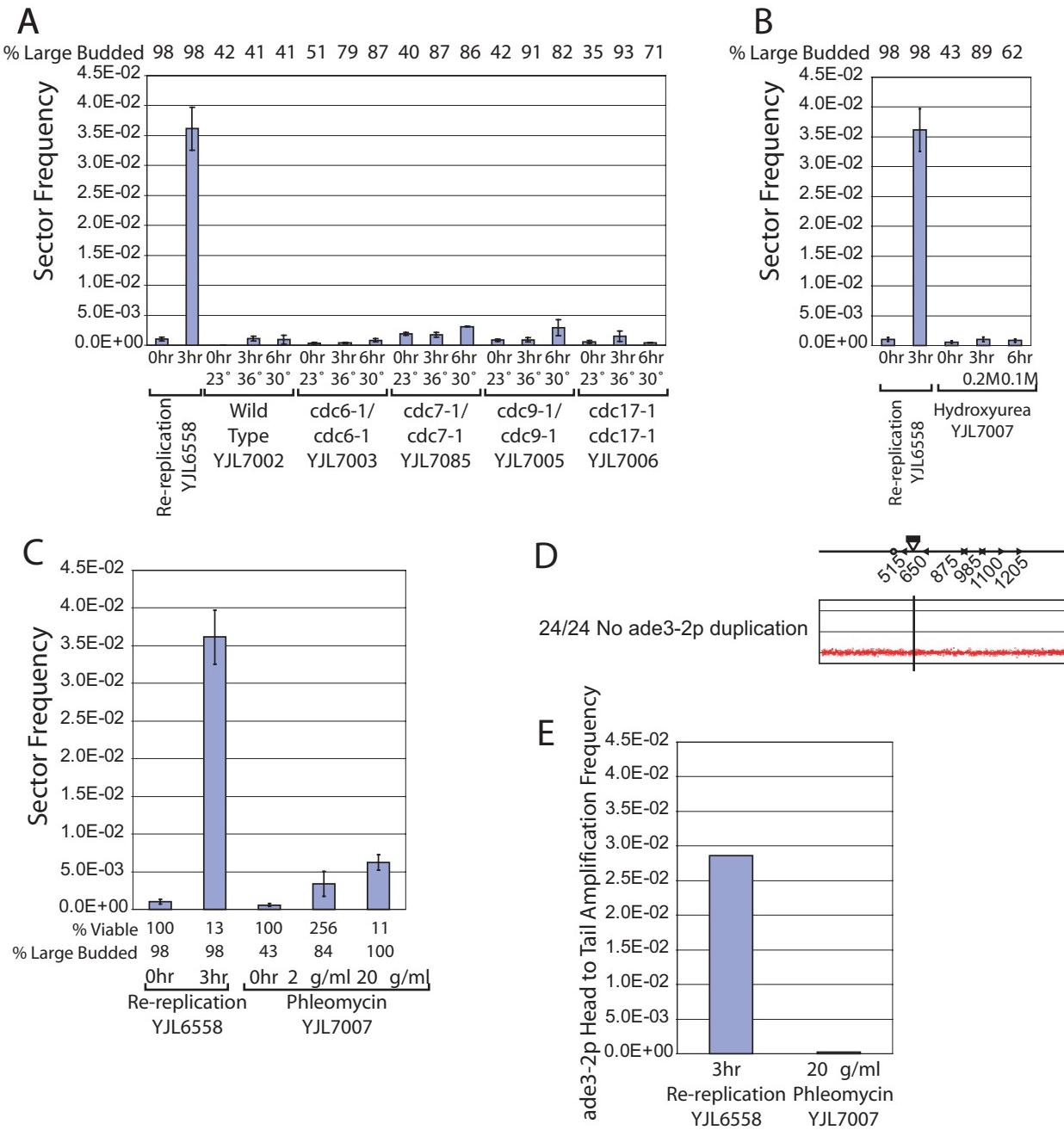
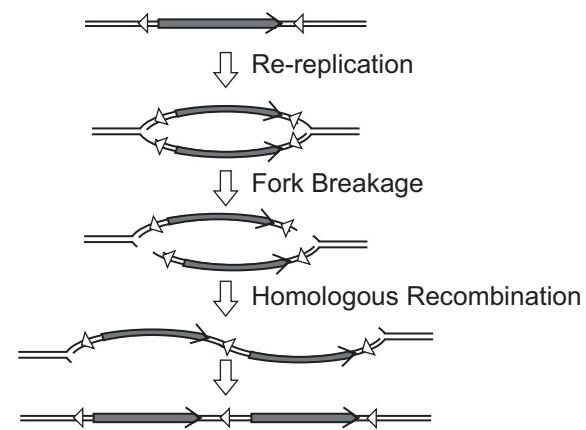


Figure 4



Supplemental Methods

Plasmids

All plasmids are described in Supplemental Table 2. Plasmids pBJL2890 and pBJL2892 consist of the following fragments of DNA: Homology Left (SacI to StuI of PCR product from OJL1796 and OJL1797 for pBJL2890 and OJL1804 and OJL1805 for pBJL2892), kanMX6 (StuI to XmaI of pFA6a-pGAL1-3HA¹), *ade3-2p* (XmaI to XbaI of pDK243²), *ARS317* (SpeI to XbaI of PCR product from OJL1794 and OJL1795 cloned into pCR2.1 TA TOPO), Homology Right (XbaI to NotI of PCR product from OJL1798 and OJL1799 for pBJL2890 and OJL1806 and OJL1807 for pBJL2892) and vector backbone (NotI to SacI of pRS56). Plasmids pBJL2889 and pBJL2891 consist of the same fragments except they lack the *ARS317* fragment.

Strains

All strains are described in Supplemental Table 1. YJL6555, YJL6557, YJL6558 and YJL6561 were generated from YJL3758 (*Mat a ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52::{pGAL-delntcdc6-cdk2A(6,8), URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1::LEU2*) by the integration of SacI to SalI from pBJL2889, pBJL2890, pBJL2891 or pBJL2892 followed by disruption of the endogenous ARS317 with a PCR product of natMX from pAG25³ with OJL1639 and OJL1640. YJL6974 and YJL6977 were generated from YJL3756 (*ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52::{pGAL, URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1::LEU2*) by the integration of SacI to SalI from pBJL2890 or pBJL2892 followed by disruption of the endogenous ARS317 with a PCR product of natMX from pAG25³ with OJL1639 and OJL1640. YJL7007 was generated

from a mating of YJL3519 (*Mat @ ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52 trp1-289 ade2 ade3 MCM7 bar1::LEU2*) to YJL3516 (*Mat a ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52 trp1-289 ade2 ade3 MCM7 bar1::LEU2*) after integration of SacI to SalI from pBJL2890 into YJL3516. YJL7002 was generated from a mating of 4541-8-1⁴ (*Mat @ leu2 ade2 ade3 his7 ura1 can1 sap3 gal1*) integrated with SacI to SalI of pBJL2980 to 4541-8-1 switched to *Mat a* with pSB283⁵. YJL7003 was generated similarly to YJL7002, but 4525-061⁴ (*Mat @ cdc6-1 leu2 ade2 ade3 can1 sap3 his7 gal1*) was used instead of 4541-8-1. YJL7005 was generated similarly to YJL7002, but 4528-091⁴ (*Mat @ cdc7-1 leu2 ade2 ade3 can1 sap3 ura1 his7 gal1*) was used instead of 4541-8-1. YJL7006 was generated similarly to YJL7002, but 4532-171⁴ (*Mat @ cdc17-1 leu2 ade2 ade3 can1 sap3 ura1 his7 gal1*) was used instead of 4541-8-1. YJL7085 was generated similarly to YJL7002, but 4524-1-3⁴ (*Mat @ cdc7-1 leu2 ade2 ade3 can1 sap3 ura1 his7 gal1*) was used instead of 4541-8-1.

Array CGH screening of sectors

DNA from 1.5ml of saturated YEPD culture was prepared using the MasterPure Yeast DNA Purification Kit (Epicentre). 40ul (80%) of each DNA sample was labeled with Cy5 and 1.5ug of purified DNA from YJL6032 was labeled with Cy3 essentially as described⁶ but samples were cleaned up as described (Pleiss, in press). Samples were hybridized as described⁶.

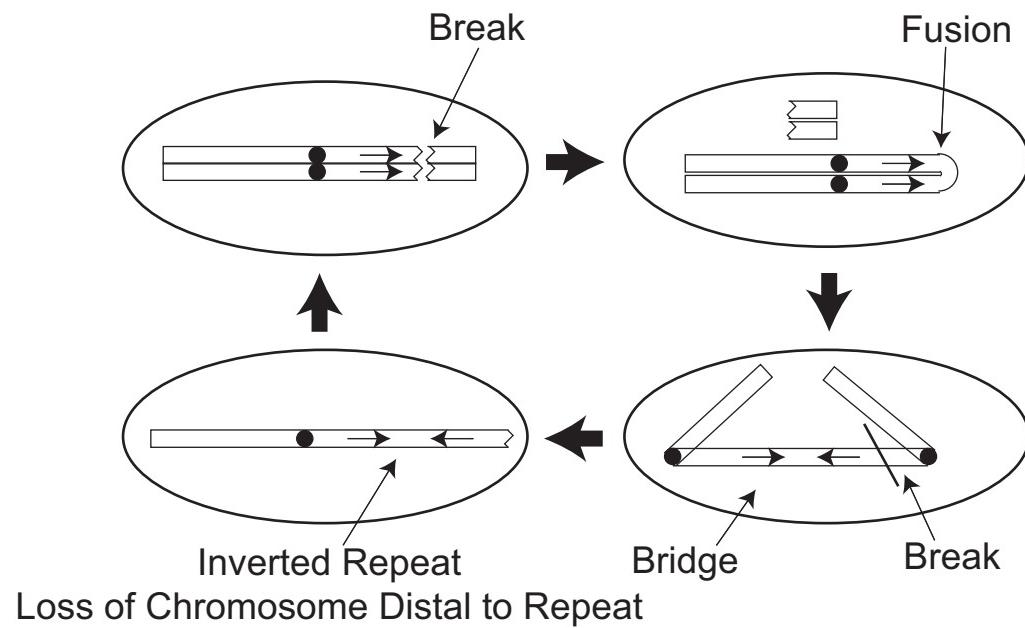
Junction PCR

To analyze novel junctions by PCR, DNA was prepared from 1.5ml of saturated culture using a modified Winston Hoffman DNA prep. Cells were pelleted in a screw cap tube and resuspended in 200ul of Winston-Hoffman Lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris.Cl pH8.0, 1mM EDTA pH8.0). 200 ul of glass beads and 200 ul of phenol/chloroform were added and the tubes were vortexed in a Tomy multi mixer (setting of 7) for 10 minutes at room temperature. 450 ul 1x TE was added to each tube, they were mixed well and spun in a microfuge at top speed for 3 minutes. 500 ul of the top layer was transferred to new screw cap tubes containing 10 ul of RNase A (10mg/ml) and incubated at 23°C for 2 hours. 300 ul of phenol/chloroform was added to each tube, they were vortexed in Tomy mixer for 5 minutes and then spun again at top speed for 3 minutes. 400 ul of the top layer was transferred to new Eppendorf tubes containing 300 ul chroloform, vortexed, and spun at top speed for 3 minutes. 300 ul of the top layer was transferred to new Eppendorf tubes containing 3 ul 10N ammonium acetate pH7.0 and 750 ul 100% ethanol. Tubes were vortexed well, then spun at top speed for 7 minutes. The DNA pellet was washed with 300ul of 70% ethanol, dried and resuspended 15 ul TE. 0.5ul of DNA was subjected to PCR with 2.5ul Roche Long Template Buffer, 1.25ul 10uM of each oligo described in Supplemental Table 3, 2.5ul 5mM dNTPs, 0.25 Roche Expand polymerase and up to 25ul H₂O. The conditions were 94°C for 3m, then 30 cycles of 94°C for 30s, 60°C for 1m, 68°C for 15m, and finally 68°C for 10m.

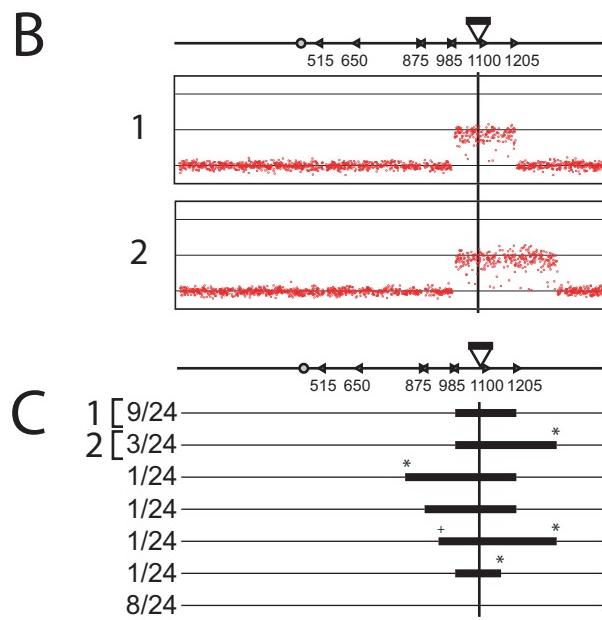
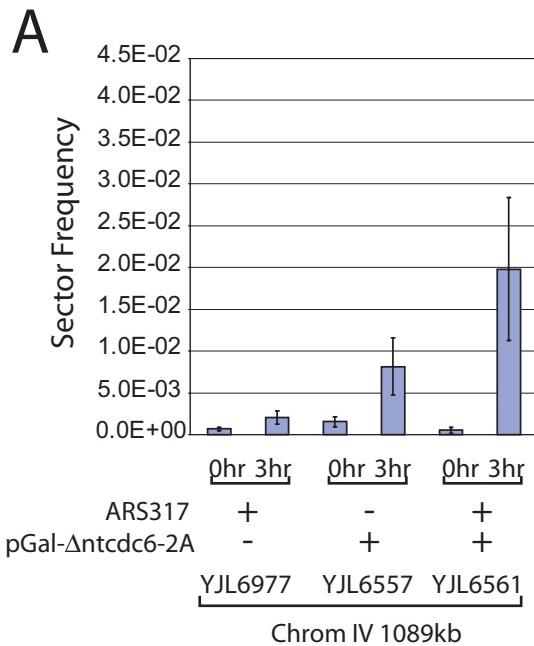
Supplemental References

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Supplemental Figure 1



Supplemental Figure 2



C

Supplemental Table 1 – Strains used in this study

YJL		Source
Number	Genotype	
YJL6555	ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52::{pGAL-delntcdc6- cdk2A(6,8), URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1::LEU2 ChromIV 567kb::{ade3-2p, kanMX} ars317::natMX	This study
YJL6557	ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52::{pGAL-delntcdc6- cdk2A(6,8), URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1::LEU2 ChromIV 1089kb::{ade3-2p, kanMX} ars317::natMX	This study
YJL6558	ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52::{pGAL-delntcdc6- cdk2A(6,8), URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1::LEU2 ChromIV 567kb::{ade3-2p ARS317, kanMX} ars317::natMX	This study
YJL6561	ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52::{pGAL-delntcdc6- cdk2A(6,8), URA3} trp1-289 ade2 ade3 MCM7-2NLS bar1::LEU2 ChromIV 1089kb::{ade3-2p ARS317, kanMX} ars317::natMX	This study
YJL6974	ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52::{pGAL, URA3} trp1- 289 ade2 ade3 MCM7-2NLS bar1::LEU2 ChromIV 567kb::{ade3-2p ARS317, kanMX} ars317::natMX	This study

	ORC2-(NotI, SgrAI) ORC6 leu2 ura3-52::{pGAL, URA3} trp1-	
YJL6977	289 ade2 ade3 MCM7-2NLS bar1::LEU2 ChromIV 1089kb::{ade3-2p ARS317, kanMX} ars317::natMX	This study
	leu2/leu2 ade2/ade2 ade3/ade3 gal1/gal1 ura1/ura1 his7/his7	
YJL7002	sap3/sap3 can1/can1 ChromIV 567kb/ChromIV 567kb::{ade3- 2p ARS317, kanMX}	This study
	cdc6-1/cdc6-1 leu2/leu2 ade2/ade2 ade3/ade3 his7/his7	
YJL7003	gal1/gal1 can1/can1 sap3/sap3 ChromIV 567kb/ChromIV 567kb::{ade3-2p ARS317, kanMX}	This study
	cdc9-1/cdc9-1 leu2/leu2 ade2/ade2 ade3/ade3 his7/his7	
YJL7005	sap3/sap3 gal1/gal1 ura1/ura1 can1/can1 ChromIV 567kb/ChromIV 567kb::{ade3-2p ARS317, kanMX}	This study
	cdc17-1/cdc17-1 leu2/leu2 ade2/ade2 ade3/ade3 his7/his7	
YJL7006	sap3/sap3 gal1/gal1 ura1/ura1 can1/can1 ChromIV 567kb/ChromIV 567kb::{ade3-2p ARS317, kanMX}	This study
	ORC2-(NotI, SgrAI)/ORC2-(NotI, SgrAI) ORC6/ORC6	
YJL7007	leu2/leu2 ura3-52/ura3-52 trp1-289/trp1-289 ade2/ade2 ade3/ade3 MCM7/MCM7 bar1::LEU2/bar1::LEU2 ChromIV 567kb/ChromIV 567kb::{ade3-2p ARS317, kanMX}	This study
	cdc6-1/cdc6-1 leu2/leu2 ade2/ade2 ade3/ade3 his7/his7	
YJL7085	gal1/gal1 can1/can1 sap3/sap3 ChromIV 567kb/ChromIV 567kb::{ade3-2p ARS317, kanMX}	This study

Supplemental Table 2 – Plasmids used in this study

Name	Description	Source
pBJL2889	ade3-2p, kanMX6 at IV_567	This study
pBJL2890	ade3-2p, kanMX6, ARS317 at IV_567	This study
pBJL2891	ade3-2p, kanMX6 at IV_1089	This study
pBJL2892	ade3-2p, kanMX6, ARS317 at IV_1089	This study
pSB283	pGAL-HO, LEU2, URA3, CEN4	Berlin, 1991 ⁵

Supplemental Table 3 – Oligonucleotides used in this study

Name	Sequence	Purpose
OJL1639	ATTAACAATGTTGATTAAAT CGCAATTAAATACCcgatccccggtaattaa	<i>ars317::natMX</i>
OJL1640	ATTTTATGGAAGATTAAGCTCATAA CTTGGACGGGGATCcatcgatgaattcgagctg	<i>ars317::natMX</i>
OJL1757	CAAAAGCATTCAAGGTACG	<i>ADE3</i> probe
OJL1758	TCAATTGCCAATGTTGGTG	<i>ADE3</i> probe
OJL1794	gctcaaatgggttaaacACTAGTACTAAAAAA AACTG	<i>ARS317</i> for cloning
OJL1795	gctcaaatgggttaaacCCAGGAGTACCTGCG CTTAT	<i>ARS317</i> for cloning
OJL1796	gctcaaatggaagcttaggcctGTTGGTGTGGTA AAGAAAA	Homology Left for pBJL2889 and pBJL2890
OJL1797	gctcaaatggagctTACAAAATTGGGGAT CATGG	Homology Left for pBJL2889 and pBJL2890
OJL1798	gctcaaatggcgccgcAAATGCCTGAGA GTTAGCC	Homology Right for pBJL2889 and pBJL2890
OJL1799	gctcaaatggaagcttctagaAGGTGTAGGCTC AAAACATA	Homology Right for pBJL2889 and pBJL2890
OJL1804	gctcaaatggaagcttaggcctGAATAAACAGAC ACTTCCTG	Homology Left for pBJL2891 and pBJL2892

OJL1805	gctcaaatgggagtcATGGAGGAACCTAAGC CTTC	Homology Left for pBJL2891 and pBJL2892
OJL1806	gctcaaatggcgccgcGAGGAGGATCACTT CTGCC	Homology Right for pBJL2891 and pBJL2892
OJL1807	gctcaaatggaagcttctagaATAGGTGAGGGA ACACCTCA	Homology Right for pBJL2891 and pBJL2892
OJL1955	TCATGCTTTGAGTAACGGTAATGA CATACATTAGTGAC	Primer 1 for junction PCR
OJL1956	CTCTTCTTACAGAAATACAAAAGGC ATGCTGATTGTTGG	Primer 2 for junction PCR
OJL1957	ACTGATGGTTCAACAGAGAAGCCAC AGTTAAAAAAGGTCC	Primer 3 for junction PCR (all sectors but Figure 2B class 4)
OJL1958	TAGGAAAACGTACTGTGATTGAAAT ACACTGGAATAGGG	Primer 4 for junction PCR (all sectors but Figure 2B class 4)
OJL1983	TTCACGATCCAAGCACTATTGCCAT TTTGTGCCCTTC	Primer 3 for junction PCR (Figure 2B class 4)
OJL1984	GCGAGGCAGGCACCTAGTCTCTAAC CCTTCATATTGATC	Primer 4 for junction PCR (Figure 2B class 4)

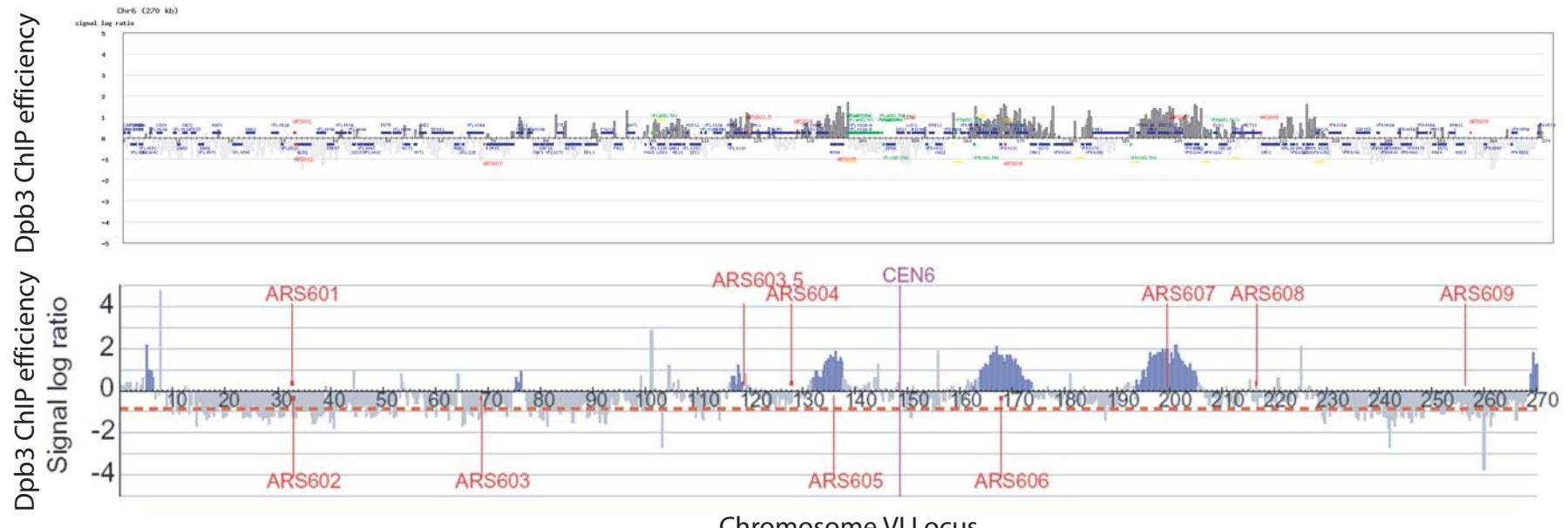
Supplemental Table 4 - Details of sectors characterized by microarray

Sector YJL#	Parent	PFGE Lane	
YJL7095	YJL6558	Fig 2C Lane 2	ade3-2p Duplication
YJL7096	YJL6558	Fig 2C Lane 3	Segmental duplications 515kb to 650kb and 515kb to 670kb
YJL7097	YJL6558	Fig 2C Lane 4	Segmental duplication 515kb to 650kb
YJL7098	YJL6558	Fig 2C Lane 5	Segmental duplication 515kb to 650kb
YJL7099	YJL6558	Fig 2C Lane 6	Segmental duplication 515kb to 650kb
YJL7100	YJL6558	Fig 2C Lane 7	No ade3-2p duplication
YJL7101	YJL6558	Fig 2C Lane 8	Segmental duplication 515kb to 650kb
YJL7102	YJL6558	Fig 2C Lane 9	Segmental duplication 515kb to 650kb
YJL7103	YJL6558	Fig 2C Lane 10	Segmental duplication 515kb to 650kb
YJL7104	YJL6558	Fig 2C Lane 11	Segmental duplication 515kb to 650kb
YJL7105	YJL6558	Fig 2C Lane 12	Segmental duplication 515kb to 650kb
YJL7106	YJL6558	Fig 2C Lane 13	No ade3-2p duplication
YJL7107	YJL6558	Fig 2C Lane 14	Segmental duplication 515kb to 650kb
YJL7108	YJL6558	Fig 2C Lane 15	Segmental duplication 515kb to 650kb
YJL7109	YJL6558	Fig 2C Lane 16	Segmental duplication 515kb to 650kb
YJL7110	YJL6558	Fig 2C Lane 17	Segmental duplication 515kb to 985kb
YJL7111	YJL6558	Fig 2C Lane 18	Segmental duplication 515kb to 650kb
YJL7112	YJL6558	Fig 2C Lane 19	Segmental duplications 515kb to 650kb and 875kb to 985kb
YJL7113	YJL6558	Fig 2C Lane 20	No ade3-2p duplication
YJL7114	YJL6558	Fig 2C Lane 21	Segmental duplication 515kb to 650kb
YJL7115	YJL6558	Fig 2C Lane 22	Segmental duplication 515kb to 650kb
YJL7116	YJL6558	Fig 2C Lane 23	Segmental duplication 515kb to 650kb
YJL7117	YJL6558	Fig 2C Lane 24	Segmental duplication 515kb to 650kb
YJL7118	YJL6558	Fig 2C Lane 25	No ade3-2p duplication
YJL7119	YJL6561		Segmental duplication 805kb to 1205kb
YJL7120	YJL6561		Segmental duplication 985kb to 1205kb
YJL7121	YJL6561		Segmental duplication 875kb to 1205kb
YJL7122	YJL6561		Segmental duplication 985kb to 1205kb
YJL7123	YJL6561		Segmental duplication 985kb to 1205kb
YJL7124	YJL6561		No ade3-2p duplication
YJL7125	YJL6561		No ade3-2p duplication
YJL7126	YJL6561		Segmental duplication 985kb to 1350kb
YJL7127	YJL6561		Segmental duplication 985kb to 1205kb
YJL7128	YJL6561		Segmental duplication 985kb to 1205kb
YJL7129	YJL6561		Segmental duplication 985kb to 1205kb
YJL7130	YJL6561		No ade3-2p duplication
YJL7131	YJL6561		Segmental duplication 985kb to 1205kb
YJL7132	YJL6561		No ade3-2p duplication
YJL7133	YJL6561		Segmental duplication 985kb to 1205kb
YJL7134	YJL6561		No ade3-2p duplication
YJL7135	YJL6561		Segmental duplication 985kb to 1350kb
YJL7136	YJL6561		Segmental duplication 985kb to 1350kb
YJL7137	YJL6561		Segmental duplication ~925kb to 1350kb
YJL7138	YJL6561		No ade3-2p duplication
YJL7139	YJL6561		Segmental duplication 985kb to 1205kb
YJL7140	YJL6561		No ade3-2p duplication
YJL7141	YJL6561		No ade3-2p duplication
YJL7142	YJL6561		Segmental duplication 985kb to 1150kb
YJL7143	YJL7007		No ade3-2p duplication

YJL7144	YJL7007	No ade3-2p duplication
YJL7145	YJL7007	No ade3-2p duplication
YJL7146	YJL7007	No ade3-2p duplication
YJL7147	YJL7007	No ade3-2p duplication
YJL7148	YJL7007	No ade3-2p duplication
YJL7149	YJL7007	No ade3-2p duplication
YJL7150	YJL7007	No ade3-2p duplication
YJL7151	YJL7007	No ade3-2p duplication
YJL7152	YJL7007	No ade3-2p duplication
YJL7153	YJL7007	No ade3-2p duplication
YJL7154	YJL7007	No ade3-2p duplication
YJL7155	YJL7007	No ade3-2p duplication
YJL7156	YJL7007	No ade3-2p duplication
YJL7157	YJL7007	No ade3-2p duplication
YJL7158	YJL7007	No ade3-2p duplication
YJL7159	YJL7007	No ade3-2p duplication
YJL7160	YJL7007	No ade3-2p duplication
YJL7161	YJL7007	No ade3-2p duplication
YJL7162	YJL7007	No ade3-2p duplication
YJL7163	YJL7007	No ade3-2p duplication
YJL7164	YJL7007	No ade3-2p duplication
YJL7165	YJL7007	No ade3-2p duplication
YJL7166	YJL7007	No ade3-2p duplication

Figure 1

Our results (no averaging)



Katou et al, 2003 (average of multiple experiments)

Chromatin immunoprecipitation can be performed in our laboratory with collaboration with the lab of Dr. Shirahige. We prepared DNA from cells arrested in S phase and used chromatin immunoprecipitation against Dpb3 to enrich for early replicating sequences of DNA. We sent this DNA to Dr. Shirahige and his lab determined the amount of DNA for each chromosomal locus on chromosome VI. Both our results and averaged, published results are shown.